Figure 20 shows a perfect example of the single corner flapping (7) as suggested by Ōsawa;[24] i.e., the corner is flapped all the way to the opposite side of the plane of the ring.

Conclusions

We have demonstrated that contemporary computational methods and resources allow more to be accomplished in the field of conformational analysis than simply collecting low minimum energy conformers. It is possible to search conformational space in a more comprehensive manner, namely, to determine the complete network of conformational interconversions (NCI) within a reasonable energy window. The key is to search for saddle points on the potential energy hypersurface, rather than for minima. Although there is no simple computational technique available that converges exclusively upon saddle points, the use of a combination of the truncated Newton conjugate gradient and the full-matrix Newton–Raphson minimization methods allowed us to find saddle points at a comparable rate to finding minima. It was shown that the pair of minima associated with a particular saddle point can be found using a straightforward procedure. It was further shown that these so-called minimum-saddle-minimum (MSM) triads, each representing an individual conformational interconversion, can be fit together to solve the jigsaw puzzle of the NCI. The NCI can be visualized on a three-dimensional staircase diagram. Although the search for the NCI is obviously more computationally intensive than the traditional search for only minima, our results indicate that, using a reasonable energy window, it can be accomplished for small- to medium-ring cycloalkanes using a reasonable amount of CPU time on modern workstations.

The C_4–C_{12} cycloalkane series has been subjected to comprehensive conformational analysis with MM2 steric energies. Results derived from each NCI were in agreement with literature data on well-known conformational interconversions in the C_4–C_{12} series. Moreover, our analysis revealed that the different modes of conformational interconversion associated with the lowest barriers between minimum energy conformers display fascinating patterns of well-coordinated atomic movement. It was found that 97% of the 115 conformational interconversions in cyclohexane to cyclododecane exhibit the same basic atomic movements. Indeed, each interconversion, no matter how complex, can be decomposed into a few basic modes. There are two such basic modes termed kayaking and flapping. Kayaking involves four different modes, two of them involving three ring bonds, and the other two modes involving five ring bonds. Furthermore, both the three- and the five-bond kayaking modes have two versions, one through an eclipsed and another through a trans central torsion angle in the transition state. Flapping can be single or double corner flapping, and handle flapping. Flapping modes (in any combination) exhibit an alternating inward–outward pattern; i.e., corners and/or handles are flapped in an "in–out–in–out" fashion all around the ring. It is instructive to note that no distinction can be made between interconversions between different conformers and interconversions between identical forms or enantiomers of the same conformer in terms of modes of interconversion. Both types exhibit all basic modes of interconversion, but the interconversions between identical conformers and between enantiomers are usually more symmetrical. We believe that the kayaking and flapping modes are not artifacts of the MM2 force field. Our results suggest that kayaking and flapping are as basic to cycloalkane interconversions as torsional rotation is to *n*-alkane interconversions.

Acknowledgment. We are indebted to a reviewer for suggestions and for giving recent references to advanced saddle point search techniques.

Supplementary Material Available: Table of the MM2 steric energies and the torsion angles of all the minimum energy conformers and transition states corresponding to the MSM triads in Table II (12 pages). Ordering information is given on any current masthead page.

Nucleic Acid Dendrimers: Novel Biopolymer Structures†

Robert H. E. Hudson and Masad J. Damha*,[1]

Contribution from the Department of Chemistry, University of Toronto, Erindale College, Mississauga, Ontario, Canada L5L 1C6. *Received July 9, 1992*

Abstract: A general convergent-growth procedure for the synthesis of nucleic acid dendrimers has been developed. The synthetic strategy involves (i) the synthesis of oligonucleotides on the surface of controlled-pore glass with an automated DNA synthesizer, (ii) the introduction of the branch-point nucleoside by the coupling of two adjacent polymer-bound nucleotide chains with a tetrazole-activated adenosine 2′,3′-bis(phosphoramidite) derivative, and (iii) repetitive chain elongation and branching steps to form successive generations (G = 1–3), each with twice as many chain ends as the previous generation. Various dendrimers were constructed based on thymidine and adenosine building blocks, including an 87-unit-long dendrimer having a molecular weight of ca. 25 000 with six branch points and twelve terminal ends at the periphery of the macromolecule. Full enzymatic characterization of the dendrimers is also described.

Introduction

The past few years have been marked by research directed toward the design of highly branched macromolecules with well-defined molecular composition and constitution.[2] Since creating the first "starburst" dendrimers, the poly(amidoamines), or PAMAMs,[3] Tomalia and co-workers have synthesized a number of dendrimer families, some possessing striking similarities to anionic micelle systems.[1,4] Several other novel applications of Tomalia's "divergent-growth" approach have recently appeared, leading to dendritic poly(amidoalcohols), or "arborols",[5] polyamines,[6] poly(siloxysilanes),[7] and the micelle-like polyphenylenes[8]

† Dedicated to Professor Kelvin Kenneth Ogilvie on the occasion of his 50th birthday.

(1) Present address: Otto Maass Chemistry Building, Department of Chemistry, McGill University, Montreal, P.Q., Canada H3A 2K6.

(2) Extensive reviews of this area have appeared: (a) Tomalia, D. A.; Naylor, A. M.; Goddard, W. A., III. *Angew. Chem., Int. Ed. Engl.* **1990**, *29*, 138. (b) Tomalia, D. A.; Hedstrand, D. M.; Wilson, L. R. *Encyclopedia of Polymer Science and Engineering*, 2nd ed.; J. Wiley: New York, 1990; index volume, pp 46–92. (c) Tomalia, D. A. *New Sci.* **1991**, Nov 23, 30–34.

(3) Tomalia, D. A.; Baker, H.; Dewald, J.; Hall, M.; Kallos, G.; Martin, S.; Roeck, J.; Smith, P. *Polym. J. (Tokyo)* **1985**, *17*, 117.

(4) Gopidas, K. R.; Leheny, A. R.; Caminati, G.; Turro, N. J.; Tomalia, D. A. *J. Am. Chem. Soc.* **1991**, *113*, 7335.

0002-7863/93/1515-2119$04.00/0

and "alkane cascade" polymers.[9] Fréchet and co-workers have also developed elegant "convergent-growth" approaches to polyether and polyester macromolecules based on substituted phenols and aroyl chloride repeating units.[10]

Our interest in the biological role of branched ribonucleic acids (bRNA), intermediates of RNA splicing reactions in eukaryotic cells,[11] initially led us to synthesize branched oligomers consisting of (3′–5′) linked nucleotide units with a single adenosine (2′–5′) branching point.[12] We report here an extension of our methodologies, which utilizes standard phosphoramidite chemistry[13] and a "convergent-growth" approach, that enables one to synthesize hyperbranched polymer structures based on multifunctional nucleotide building blocks.[14]

Experimental Section

Reagents. Reagent grade aqueous ammonia, acetic anhydride (Ac_2O), trichloroacetic acid (TCA), 1,2-dichloroethane (DCE), iodine (BDH, Toronto, ON), *N*-methylimidazole (*N*-MeIm, Aldrich Chemical Company, Milwaukee, WI), and DNA synthesis grade tetrazole (Dalton Chemical Laboratories, DCL, Toronto, ON) were used as received. Diethyl ether (BDH) was stored over activated molecular sieves (methanol washed, 400 °C/24 h; Linde 4A). Pyridine, lutidine, and hexanes (BDH) were distilled from calcium hydride (BDH) and stored over molecular sieves. Water was double distilled in glass, treated with diethyl pyrocarbonate (Aldrich), and autoclaved (30 min, 121 °C, 5 atm). High-performance liquid chromatography (HPLC) grade methanol and AnalaR KH_2PO_4 were obtained from BDH. Polyacrylamide gel electrophoresis (PAGE) reagents were purchased from Bio-Rad. Sephadex G-25 (Pharmacia, Baie d'Urfé, PQ) and reversed-phase chromatography cartridges (C18 SEP-PAK, Waters, Mississauga, ON) were used according to the manufacturer's specifications.

Oligonucleotide Synthesis: General Considerations. Reagents for the solid-phase synthesis of oligonucleotides were purchased from Applied Biosystems Inc. (ABI, Mississauga, ON) and DCL or were prepared as below. Anhydrous acetonitrile was predried by distillation from P_2O_5 (BDH) and redistilled from calcium hydride under dry argon (Canox, Bramalea, ON). Tetrahydrofuran (BDH) was predried over KOH pellets, filtered, and distilled immediately prior to use from sodium benzophenone ketyl. Detritylation was performed with 3% trichloroacetic acid in DCE. Activation of phosphoramidite reagents was achieved with 0.5 M tetrazole in acetonitrile. Reagents for acetylation (capping) were prepared as follows: Cap A, 10% acetic anhydride and 10% 2,6-lutidine in THF; and Cap B, 16% *N*-methylimidazole in THF (w/v). Iodine, 0.1 M, in THF/pyridine/water (25:20:2, v/v/v) was used for oxidations. 5′-*O*-(Dimethoxytrityl)thymidine 3′-*O*-(2-cyanoethyl *N,N*-diisopropylphosphoramidite) was purchased from ABI and DCL or was prepared by standard protocol. N^6-Benzoyl-5′-*O*-(dimethoxytrityl)adenosine 2′,3′-*O*-bis(2-cyanoethyl *N,N*-diisopropylphosphoramidite) was prepared according to the method of Damha and Ogilvie.[15] These phosphoramidite reagents were stored at −20 °C and desiccated under vacuum and over P_2O_5 for 24 h prior to use. Long-chain-alkylamine controlled-pore glass (LCAA-CPG, 500-Å pore size; density: ca. 0.4 g/mL) was obtained from Pierce Chemical Co. (Rockford, IL) or CPG, Inc. (Fairfield, NJ) and derivatized according to a literature procedure[16] with the exception of prolonged acid activation. Activation of commercial LCAA-CPG by stirring over 5% TCA in DCE (w/v) for 24–72 h was found to be necessary to achieve the desired nucleoside loading in the subsequent derivatization steps. 5′-*O*-(Dimethoxytrityl)thymidine nucleoside loadings of 33–47 μmol/g were attained, as determined by spectrophotometric dimethoxytrityl cation assay.

Automated Oligonucleotide Synthesis. Dendron synthesis was carried out on an Applied Biosystems 381A synthesizer using a slightly modified 1-μmol-scale pulsed-delivery cycle in the "trityl off" mode. The 1-μmol-scale β-cyanoethyl cycle supplied by ABI was modified in the following manner: (a) *phosphoramidite coupling*, a 300-s "wait" for 5′-*O*-(dimethoxytrityl)thymidine 3′-*O*-(2-cyanoethyl *N,N*-diisopropylphosphoramidite) and a 600-s "wait" for N^6-benzoyl-5′-*O*-(dimethoxytrityl)adenosine 2′,3′-*O*-bis(2-cyanoethyl *N,N*-diisopropylphosphoramidite); (b) *acetylation* (*capping*), a 20-s delivery of "cap A" and "cap B" to the column followed by a 30-s "wait"; (c) *oxidation*, a 30-s delivery of iodine solution to the column followed by a 20-s "wait"; and (d) *detritylation*, a 120-s delivery to the column (of TCA/DCE solution) for DMT deblocking and a 180-s delivery for MMT deblocking. Detritylation times may be reduced to ca. 60 s for oligomers containing N^6-benzoyldeoxyadenosine.

Prior to oligonucleotide assembly, the derivatized support was treated with capping reagents to block undesired reactive sites[16] by use of the capping cycle provided by ABI.

5′-*O*-(Dimethoxytrityl)thymidine 3′-*O*-(2-cyanoethyl *N,N*-diisopropylphosphoramidite) and N^6-benzoyl-5′-*O*-(dimethoxytrityl)adenosine 2′,3′-*O*-bis(2-cyanoethyl *N,N*-diisopropylphosphoramidite) were dissolved in dry, freshly distilled acetonitrile to concentrations of 0.15 M and 0.025 M, respectively. All solutions were passed through a 0.45-μm-pore Teflon filter (Millipore, Mississauga, ON) before placement on the automated synthesizer. The support was dried in vacuo 24 h before use, loaded into an empty column with replaced filters (ABI), crimped closed with aluminum seals (Pierce), and installed on the instrument. The syntheses were evaluated by UV spectroscopic quantitation of trityl cation released during the trichloroacetic acid treatment step [504 nm for DMT+ (T additions) and 478 nm for MMT+ (branch-point additions)]. Under optimal conditions, the absorbances of trityl cations released during T addition steps were ca. 1.5–2.5-fold greater than for those released in the immediate A branching step.

Standard deprotection conditions were employed (29% NH_4OH, 24 h at room temperature) to liberate the oligomers from the solid support as well as to remove the exocyclic amino blocking groups. The solutions of oligomers were lyophilized, and the amount of material was quantitated by the UV absorbance at λ = 260 nm. The oligomers were purified by PAGE (24%) utilizing both denaturing (7 M urea) and non-denaturing conditions. Following electrophoresis, the gels were covered with Saran Wrap and photographed over a fluorescent TLC plate (Merck, distributed by EM Science, Gibbstown, NJ) illuminated by a hand-held UV lamp using Polaroid PolaPan 4 × 5 in^2. Instant Sheet Film (#52, medium contrast, ISO 400/21 °C; f4.5, 16 s) through a Kodak Wratten gelatin filter (#58). Subsequently, the oligomers were water extracted from crushed gel and desalted by reversed-phase chromatography (C18 SEP-PAK) or via size-exclusion chromatography (Sephadex G-25). The purified oligomers were obtained by evaporation to dryness (Savant Industries Speed-Vac) and quantitated.

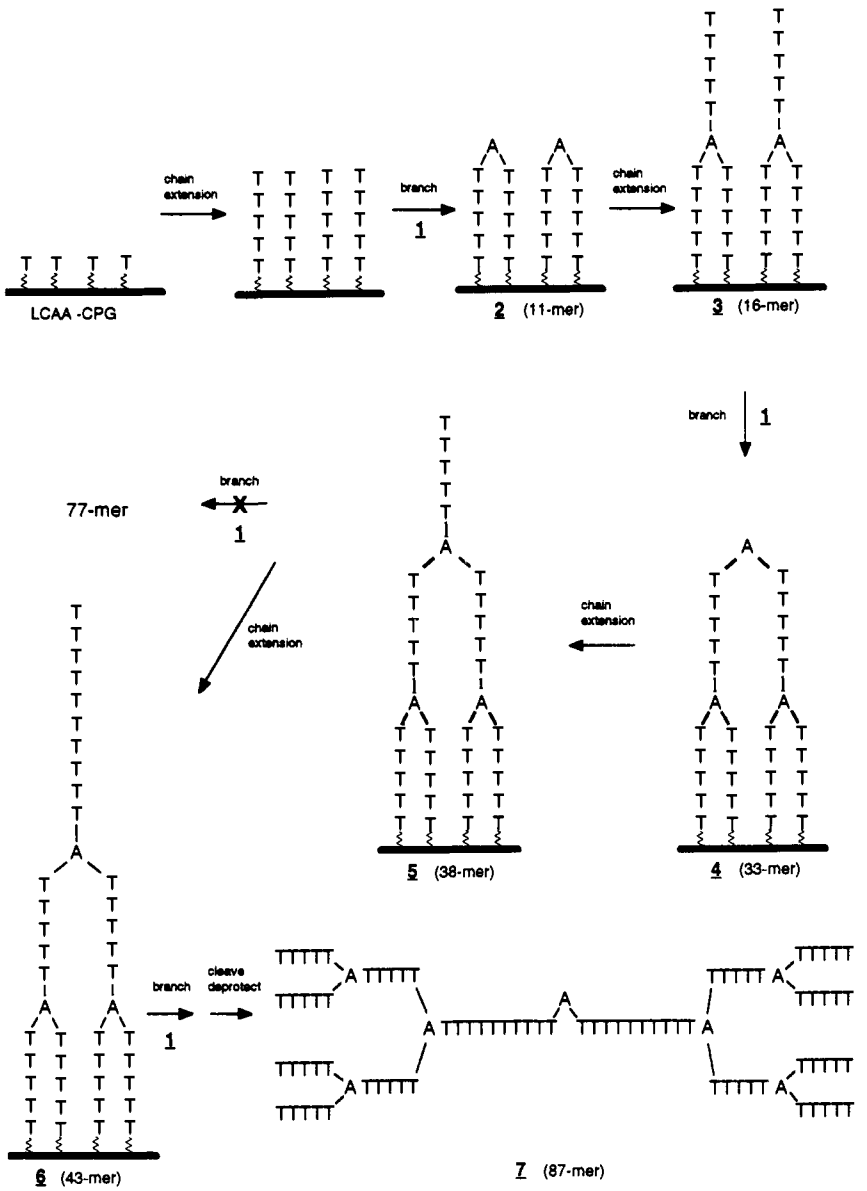
Enzymatic Hydrolysis of Oligonucleotides. (i) Snake venom phosphodiesterase (SVPDE) from *Crotalus durissus* (Boehringer Mannheim, Dorval, PQ) was obtained as a solution of 2 mg/mL in 50% (v/v) glycerol, pH ≈ 6.0. (ii) Alkaline phosphatase (AP) from calf intestine (Boehringer Mannheim) was obtained as a suspension of 2 mg/mL in a solution containing $(NH_4)_2SO_4$ (3.2 M), $MgCl_2$ (1 mM), and $ZnCl_2$ (0.1 mM), pH ≈ 7.0. (iii) Nuclease P1 (NP1) from *Penicillium citrinum* (Boehringer Mannheim) was obtained as a lyophilized powder and was dissolved in 30 mM NH_4OAc (pH 5.3) to a concentration of 1 mg/mL. (iv) Calf spleen phosphodiesterase (CSPDE, Boehringer Mannheim) was obtained as a suspension of 2 mg/mL in $(NH_4)_2SO_4$ (3.2 M), pH ≈ 6.0.

Incubation buffers for the enzyme digestions were prepared from autoclaved water and filtered through a sterile 0.2-μm membrane (Acrodisc, Gelman Sciences, Inc., Rexdale, ON). SVPDE/AP incubations were performed in 50 mM Tris-HCl, 10 mM $MgCl_2$ (pH 8); NP1/AP incubations were carried out in 0.1 M Tris-HCl, 1 mM $ZnCl_2$ (pH 7.2); and CSPDE incubations took place in 0.5 M $(NH_4)_2SO_4$, pH ≈ 6.0.

Typically, 0.2 A_{260} unit of oligonucleotide was dissolved in 30 μL of

(5) Newkome, G. R.; Yao, Z.; Baker, G. R.; Gupta, V. K. *J. Org. Chem.* **1985**, *50*, 2003.
(6) Suzuki, M.; Iii, A.; Yoshida, S.; Saegusa, T. *Progr. Pac. Polym. Sci., Proc. Pac. Polym. Conf., 1st 1989*, **1991**, 439.
(7) (a) Uchida, H.; Kabe, Y.; Yoshino, K.; Kawamata, A.; Tsumuraya, T.; Masamine, S. *J. Am. Chem. Soc.* **1990**, *112*, 7077. (b) Mathias, L. J.; Carothers, T. W. *J. Am. Chem. Soc.* **1991**, *113*, 4043.
(8) Kim, Y. H.; Webster, O. W. *J. Am. Chem. Soc.* **1990**, *112*, 4592.
(9) (a) Newkome, G. R.; Moorefield, C. N. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 1176. (b) Newkome, G. R.; Moorefield, C. N.; Baker, G. R.; Saunders, M. J.; Grossman, S. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 1178.
(10) (a) Hawker, C. J.; Fréchet, J. M. J. *Macromolecules* **1990**, *23*, 4726. (b) Wooley, K. L.; Hawker, C. J.; Fréchet, J. M. J. *J. Am. Chem. Soc.* **1991**, *113*, 4252. (c) Hawker, C. J.; Lee, R.; Fréchet, J. M. J. *J. Am. Chem. Soc.* **1991**, *113*, 4583.
(11) Sharp, P. A. *Science* **1987**, *235*, 766.
(12) (a) Damha, M. J.; Zabarylo, S. V. *Tetrahedron Lett.* **1989**, *30*, 6295. (b) Damha, M. J.; Ganeshan, K.; Hudson, R. H. E.; Zabarylo, S. V. *Nucleic Acids Res.* **1992**, *20*, 6565.
(13) (a) Caruthers, M. H. *Science* **1985**, *230*, 281. (b) Caruthers, M. H. In *Synthesis and Applications of DNA and RNA*; Narang, S. A., Eds.; Academic Press, Inc.: Florida and London, 1987; pp 47–94.
(14) Preliminary results of this work have been communicated: Damha, M. J.; Hudson, R. H. E.; Ganeshan, K.; Zabarylo, S. V.; Guo, Y. International Symposium on Synthetic Oligodeoxynucleotides: Problems and Frontiers of Practical Application, Moscow, USSR, June 23–30, 1991; *Nucleic Acids Res. Symposium Series* **1991**, *24*, 203.
(15) Damha, M. J.; Ogilvie, K. K. *J. Org. Chem.* **1988**, *53*, 3710.
(16) Pon, R. T.; Usman, N.; Ogilvie, K. K. *BioTechniques* **1988**, *6*, 768.

Scheme I



buffer to which the appropriate enzyme was added, and the mixture was incubated at 37 °C. Digests with SVPDE were accomplished with 1 μL of enzyme (2 μg, 0.002 unit) and 1 μL of AP (6 μg, 9 units). Two microliters of NP1 (2 μg, 0.6 unit) and 1 μL of AP (6 μg, 9 units) were used to determine T:A(2′p5′T)3′p5′T ratios. Studies with CSPDE employed 2 μL (4 μg, 0.004 unit) of enzyme in 10 μL of incubation buffer. SVPDE/AP digestion was usually complete after ca. 18 h, whereas incubations with NP1/AP required longer periods (24–72 h) for total digestion. Digestions with CSPDE were performed for 120 min. CSPDE digests were analyzed by analytical PAGE. After the 2-h incubation period, 15 μL of loading buffer (8:2 deionized formamide/TBE buffer) was added to the digest, and this mixture was directly loaded onto the gel for analysis.

High-Performance Liquid Chromatography. HPLC analyses of the enzyme digestions were performed on a Waters instrument equipped with dual 501 pumps, a UK6 injector, and a 480 UV detector governed by the 680 gradient controller being output through a 480 data module. Alternatively, analyses were conducted on a similarly equipped Waters instrument using the 600E controller and solvent delivery system. The column employed was a Whatman Partisil ODS-2 (4.6 × 250 mm) with a linear gradient 0–50% in solvent B over 30 min (solvent A, 20 mM KH_2PO_4 pH 5.5; solvent B, methanol) and a 2 mL/min flow rate at room temperature.

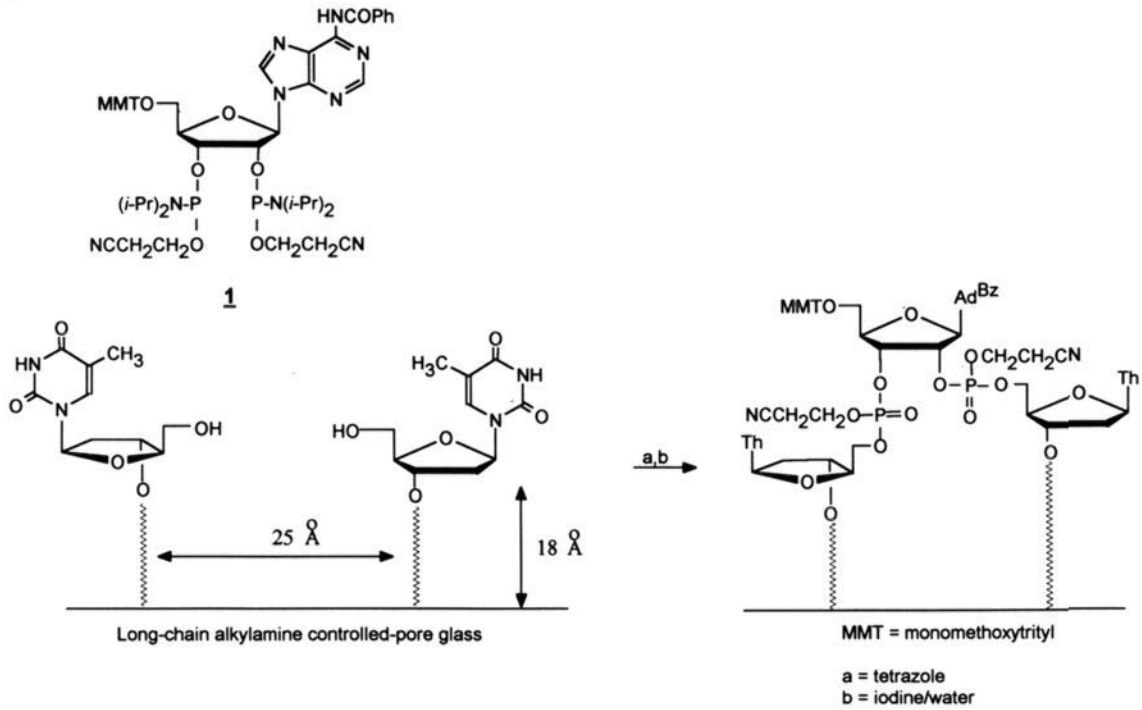
Samples were centrifuged at 14000*g* for 3 min prior to analysis in order to clear the solution of particulates. Injections of ca. 10–15 μL (0.1 A_{260}) were found to be adequate for the determinations. Components of the analyte solution were identified by comparison to authentic samples of either purchased [thymidine, adenosine, inosine (Sigma Chemical Co., St. Louis)] or previously synthesized and characterized molecules [A-(2′p5′T)3′p5′T].[15] Due to contaminant adenosine deaminase activity in commercial preparations of SVPDE, digestion with this enzyme released inosine (derived from adenosine) for the oligomers.

Capillary Electrophoresis. Oligomers were characterized on an Applied Biosystems 270A capillary electrophoresis system equipped with a Shimadzu C-R5A Chromatopak integrator and fitted with an ABI Microgel$_{100}$ capillary column. The elution buffer consisted of 75 mM Tris-phosphate, pH 7.6, and 10% methanol. Between 0.02 and 0.5 A_{260} units of material was injected in 30 μL of water.

Results and Discussion

The overall strategy is represented in Scheme I by the syntheses of various dendrimers, including an 87-unit-long structure having a molecular weight of ca. 25 000 and six branch points at the periphery of the molecule. Our strategy employs the solid-phase method for oligonucleotide synthesis and a fully protected adenosine 2′,3′-*O*-bis(phosphoramidite) reagent (**1**) to join neighboring polymer-bound oligomers together, thus forming the branch point (Scheme II). We have previously shown that the efficiency of the branching reaction is dependent on both the degree of derivatization of the solid support and the solution concentration of the bis(phosphoramidite) reagent **1**.[12] Solid supports with a high degree of substitution give the best results since they ensure an appropriate distance between the reactive end groups of the immobilized oligonucleotide chains. Long-chain-alkylamine controlled-pore glass (LCAA-CPG) is a well-known solid-phase support that fulfills this requirement[17] since it is well suited to

Scheme II



being functionalized up to 70 μmol oligonucleotide chains/g by procedures developed by Pon et al.[16] and our research group.[18] In addition, LCAA-CPG's 18-Å-long alkyl spacer makes the support-bound oligomers more accessible to coupling reagents, and its pore size (500 Å) is conducive to the synthesis of high molecular weight oligonucleotide chains. Low concentrations of bis(phosphoramidite) **1** should be employed in the branching step, since under these conditions only a fraction of the 5′-hydroxyl groups at the chain ends become phosphitylated, hence favoring the branching reaction (i.e., 5′-HO-TpTpT... → 5′-$A^{p}{}_{pTpTpT...}$ + unreacted 5′-HO-TpTpT... → branch $A^{pTpTpT...}{}_{pTpTpT...}$).

Our syntheses start from thymidine anchored to LCAA-CPG [23 mg (1.0 μmol, 47 μmol/g) of 5′-*O*-(dimethoxytrityl)thymidine–succinyl–LCAA-CPG]. $(Tp)_4T$ was assembled on the surface by the successive addition of standard deoxythymidine phosphoramidite reagent in an automated chemical process. The branch juncture was introduced in a sequence of two reactions involving branch formation [1.4 mg (1.3 μmol) of **1**, 1.9 mg (27.7 μmol) of tetrazole, 155 μL of MeCN; 10 min] followed by oxidation (I_2/H_2O) of the vicinal phosphite triester linkages to the more stable phosphate triesters (Schemes I and II). Capping (Ac_2O/*N*-MeIm) was performed after each monomer addition and branching step. Chain elongation and branching steps are repeated to form successive generations (G = 1–3, Scheme I), each with twice as many chain ends as the previous generation. Attempts to branch 38-mer–CPG **5** with **1** were unsatisfactory probably due to the large spacing that exists between the reactive (5′) end groups of neighboring dendrimers[19] and/or the steric and dynamic constraints that these experience on the CPG surface. Indeed, when the 5′ "arm" of **5** was lengthened by about 25 Å (i.e., **5** → **6**; Scheme I), the branching reaction with **1** occurred to produce an 87-unit-long dendrimer (**7**).

(17) Simple geometric calculations show that the maximum separation between oligonucleotide chains on the surface of LCCA-CPG (50 μmol/g) is ca. 25 Å. Since the length of the alkyl chain spacer which joins the oligomers to the support surface is ca. 18 Å, the 5′-end groups of oligomers can "reach" one another.

(18) Damha, M. J.; Giannaris, P. A.; Zabarylo, S. V. *Nucleic Acids Res.* **1990**, *13*, 3813.

(19) On the basis of the loading of thymidine on LCAA-CPG (47 μmol/g) and the 5′-to-3′-end distance of a thymidine 5′-monophosphate unit (ca. 4.7 Å), the separation between the terminal 5′-OH groups of neighboring dendrimers (**5**) is estimated to be ca. 8–10 Å. This distance would be too large for the 5′-OH groups to be bridged.

The oligomers were concomitantly cleaved from the support and deprotected by the standard treatment with 29% NH_4OH (24 h, room temperature). Removal of the ammoniacal solution furnished the crude dendrimers. Initial characterization and purification of dendrons was accomplished by PAGE and reversed-phase liquid chromatography. Figure 1 shows a polyacrylamide gel electrophoretogram of the crude synthesis mixture of **4**. The pattern of bands revealed in the PAGE chromatogram is found to represent a synthesis "fingerprint". As for all successful dendron preparations, the "fingerprint" pattern is characteristic of the particular synthesis and, in conjunction with the quantitation of released trityl cation, is useful in diagnosing synthetic difficulties.

The band structure, or "fingerprint", arises as a result of incomplete reaction at the branching step. For instance, in the case of **4** (Figure 1), the least mobile band has been found to be the full-length dendrimer, vide infra. Some of the "default", or more mobile, bands can be accounted for by support-bound oligomers that fail to react with **1** which are subsequently capped and prevented from further extension. Others are a result of monophosphitylation (either 3′–5′ or 2′–5′) of CPG-bound oligomers

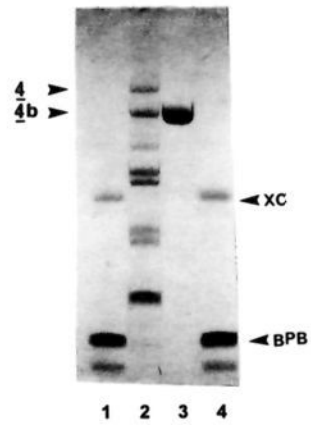


Figure 1. Polyacrylamide gel electrophoresis (24%, 7 M urea) of crude dendrimer **4** (33-mer) and a linear 33-mer of identical base composition. Lanes 1 and 4: marker dyes xylene cyanol (XC) and bromophenol blue (BPB). Lane 2: crude synthesis mixture of branched 33-mer **4**. Lane 3: $T_{10}AT_{10}AT_5AT_5$ (linear 33-mer). Compound numbering is the same as in Scheme I.

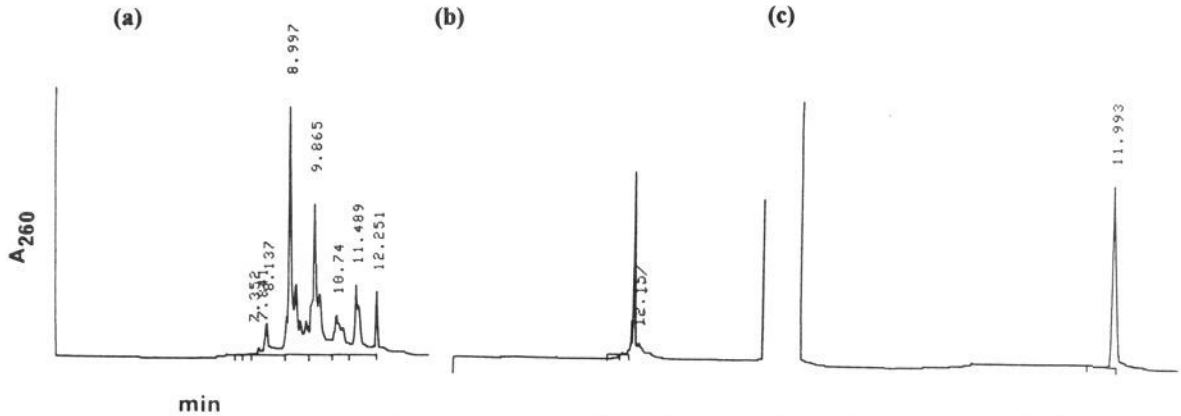


Figure 2. Characterization of **4** (33-mer) by capillary electrophoresis. (a) Capillary electrophoresis chromatogram of crude 33-mer **4**. Full-length dendrimer **4** is indicated by the peak at 12.251 min. (b) Capillary electrophoresis chromatogram of PAGE-purified **4**: 12.157 min. (c) Capillary electrophoresis chromatogram of $T_{10}AT_{10}AT_5AT_5$ (linear 33-mer): 11.993 min. Compound numbering is the same as in Scheme I.

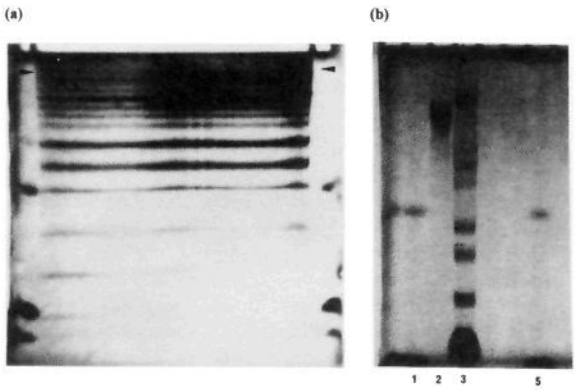


Figure 3. Characterization of **7** (87-mer) by polyacrylamide gel electrophoresis. (a) Denaturing PAGE, 24%, of crude **7**. The band selected for enzymatic digestion and HPLC analysis is marked by the arrowheads. (b) Denaturing PAGE, 12%, of crude **7**. Lane 1 and 5: marker dyes xylene cyanol and bromophenol blue. Lane 2: linear 87-unit-long oligomer $T_{10}AT_{10}AT_{10}AT_{10}AT_{10}AT_{10}AT_{10}AT_{10}$. Lane 3: crude **7**.

at the introduction of **1** followed by chain growth in the ensuing elongation steps. Figure 2, parts a, b, and c, respectively, are capillary electrophoresis (CE) chromatograms for the crude synthesis mixture of **4**, PAGE-purified **4**, and a linear oligomer of identical composition and size. The CE analysis indicates that 6.2% of the crude mixture is full-length dendrimer, whereas isolated yields by preparative PAGE are lower.

Preparative PAGE purification of the dendrimers afforded 11-mer **2** (12 A_{260} units, 23%), 16-mer **3** (10 A_{260} units, 8%), 33-mer **4** (5 A_{260} units, 2.2%), 38-mer **5** (4.5 A_{260} units, 1.7%), and 87-mer **7** (2.3 A_{260} units, 1%) of purity greater than 98% by both denaturing and non-denaturing PAGE. For comparison, linear oligodeoxynucleotides with the same base composition and number of monomer units (i.e., 11-, 16-, 33-, and 38-mers) were prepared and applied, side by side with their branched counterparts, to a 24% polyacrylamide gel. The 87-mer **7** was compared to a linear transfer RNA molecule (80-mer) isolated from *torula* yeast (24% PAGE, 7 M urea; data not shown) as well as an 87-unit-long linear oligodeoxynucleotide of analogous base composition (12% PAGE, 7 M urea; Figure 3). Relative to the linear oligomers, dendrimeric oligomers exhibited similar but retarded electrophoresis mobilities through the gel matrix, the effect being more pronounced for the more highly branched oligomers (e.g., **5** and **7**; Figures 2 and 3 and supplementary material).

As a check on the purity, structure, and nucleotide composition, a small sample of each of **2**, **3**, **4**, and **5** was subjected to enzymatic hydrolysis (nuclease P1/alkaline phosphatase, NP1/AP) and the resulting products were identified by reversed-phase HPLC (Figure 4 and supplementary material). This enzymatic digestion allows us the unique ability to selectively hydrolyze the biopolymer and explicitly determine the branched primary structure. The only

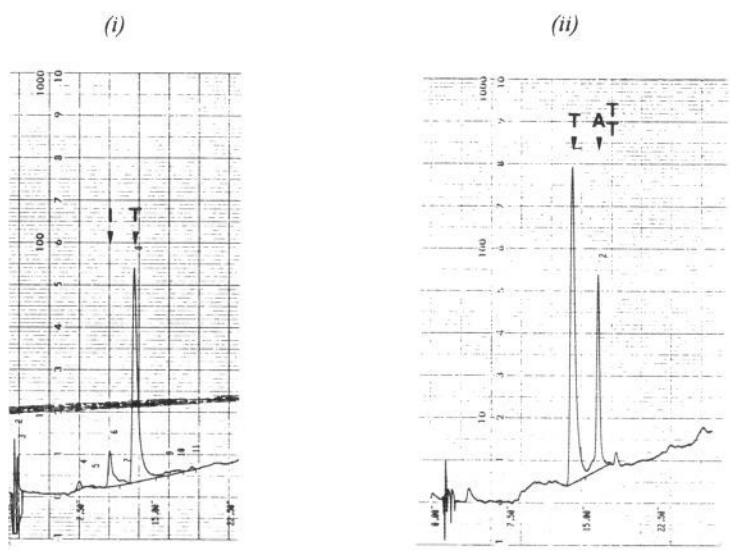


Figure 4. HPLC analysis of enzymatic digests of dendrimer **4** (33-mer). (i) Digestion of **4** (33-mer) with snake venom phosphodiesterase and alkaline phosphatase (SVPDE/AP) for compositional analysis. Observed I (from A): T ratio = 1:10.0; expected ratio = 1:10. (ii) Digestion of **4** (33-mer) with nuclease P1 and alkaline phosphatase (NP1/AP) for constitutional analysis. Observed T: A^T_T ratio = 25.3:3; expected ratio = 24:3.

Table I. Dendrimer Composition Derived from HPLC Analysis of Enzymatic Digests for Compounds 2, 3, 4, 5, and 7

	T:I ratio after SVPDE/AP[a] digestion		T:A(2′p5′dT)3′p5′dT ratio after NP1/AP[b] digestion	
compd	expected	obsd	expected	obsd
2 (11-mer)	10:1	10.0:1	8:1	8.0:1
3 (16-mer)	15:1	15.5:1	13:1	12.6:1
4 (33-mer)	30:3 (10:1)	30.0:3	24:3 (8:1)	25.3:3
5 (38-mer)	35:3	36.9:3	29:3	27.7:3
7 (87-mer)	80:7	77.5:7		

[a] Snake venom phosphodiesterase/alkaline phosphatase. [b] Nuclease P1/alkaline phosphatase.

two products detected in these digestions were unambiguously identified as thymidine and the known branched trinucleoside diphosphate A(2′p5′T)3′p5′T.[15] Integration of the peaks corresponding to these products gave the proper ratios in all cases (i.e., $T:A^{T}_{T}$ = 8:1 for 2, 13:1 for 3, 24:3 for 4, and 29:3 for 5; Table I). Progressively longer incubation periods were required for NP1/AP digestion through the series 2, 3, 4, and 5. For example, whereas a 12-h incubation period was adequate for compound 2, compound 5 required ca. 72 h under identical conditions for complete degradation. The difficulty in digesting the higher order dendrons lends additional support to the increasingly more complex, unnatural structures proposed.

In addition, treatment of dendrimers with snake venom phosphodiesterase (SVPDE) and alkaline phosphatase (AP) afforded inosine and thymidine in the predicted ratios for compounds 2, 3, 4, 5, and 7 (Figure 3 and supplementary material; inosine results from the deamination of adenosine by adenosine deaminase, which is a contaminant in the enzyme preparations). The second least mobile band in the crude mixture of 4 (4b; Figure 1) and 5 (5b; supplementary material) was also subjected to SVPDE/AP digestion. This analysis indicated compositions consistent with one incomplete branching during the first addition of 1 corresponding to the abbreviated dendrons shown in Figure 5.

Furthermore, the structures of dendrons 3 (16-mer), 5 (38-mer), and 5b (Figure 5) were correlated to those of 2, 4, and 4b, respectively, by digestion with calf-spleen phosphodiesterase. Incubation of 3 and 5 with this 5′ → 3′ exonuclease resulted in the slow removal of the five-nucleotide "5′-tail" and produced 2 (11-mer; supplementary material) and 4 (33-mer), respectively. Similarly, digestion of 5b with CSPDE resulted in the formation of 4b (Figure 5).

Conclusions

The successful synthesis and characterization of dendrimeric nucleic acids demonstrates the reliability of our general methods. With the possibility of varying the base sequence, the location of the (2′–5′) branch point, the stereochemistry of the sugar moiety, the 5′- or 3′-end modifications, and even the nature of the internucleotide linkages (e.g., phosphate → phosphorothioate), it is possible to envisage a rich and varied set of molecules which may form the basis of a new and potentially valuable class of biopolymer materials.

Current research in our laboratories is directed toward divergent, or "outburst",[2] approaches for the synthesis of nucleic acid dendrimers. The convergent approach, as demonstrated herein, is successful in yielding third-generation dendrons, but has limitations inherent in the branching strategy. The increasing amount of material present as "default" oligomers in successive generations creates a practical limit on the size of the dendrimer that can be produced. In theory, this limitation can be overcome by employing a divergent strategy, which should be capable of producing significantly increased yields of full-length dendrimer.

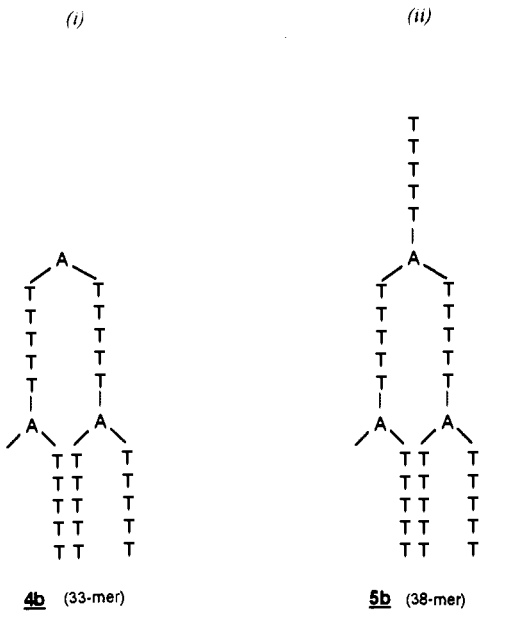


Figure 5. Proposed structures of 4b and 5b, byproducts isolated from dendrimer synthesis of 4 and 5, consistent with their composition as determined by HPLC analysis of SVPDE/AP digests. (i) Dendron 4b; see Figure 1. (ii) Dendron 5b; see the supplementary material.

Nucleic acid dendrimers have potential applications in the treatment of surfaces of conventional polymers (e.g., polycarbonates and polysulfones) to confer biocompatability. In view of their unnatural character, hyperbranched oligonucleotides also offer the exciting potential to selectively modulate gene expression via the "antisense" or "antigene" approaches.[20,21] They can also be employed as ligands for affinity purification of the branch recognition factors which catalyze the maturation or splicing of precursor messenger RNA in the cell.

Acknowledgment. We gratefully acknowledge Mr. Robert Molino of Dalton Chemical Laboratories Inc. (Toronto) for performing the capillary electrophoresis analysis. We thank Prof. M. A. Winnik (U. of Toronto) for his encouragement and helpful discussions and Prof. R. T. Pon (U. of Calgary) for generously providing linear oligodeoxynucleotide markers. We also thank the Natural Science and Engineering Council of Canada (NSERC) for financial support and a postgraduate scholarship to R.H.E.H.

Supplementary Material Available: Enzymatic characterization of the dendrimers including PAGE analysis of CSPDE incubations and HPLC chromatograms of the enzymatic digests for compounds 2, 3, 5, and 7, a comparison of the polyacrylamide gel electrophoretic mobility of dendrimers 2, 3, 4, and 5 to that of linear oligomers of identical composition, and PAGE analysis of a dendron 3 digest with CSPDE (6 pages). Ordering information is given on any current masthead page.

(20) *Oligonucleotides, Antisense Inhibitors of Gene Expression*; Cohen, J. S., Ed.; MacMillan: Houndsmills and London, 1989.
(21) Hélène, C.; Toulme, J.-J. *Biochim. Biophys. Acta* **1990**, *1049*, 99.