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THE SYNTHESIS OF BRANCHED OLIGONUCLEOTIDE STRUCTURES

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New phosphoramidite reagents, tris-2,2,2-[3-(4,4'-dimethoxytrityloxy)propyloxymethyl]-ethyl-*N*,*N*-diisopropylamino-2-cyanoethylphosphoramidite, *N*-[5-(9-fluorenylmethoxycarbonyloxy)valeryl]-*N'*-[5-(4,4'-dimethoxytrityloxy)valeryl]-2-*O*-(*N*,*N*-diisopropylamino-2-cyanoethylphosphinyl)-1,3-diamino-2-propanol, and *N*-[4-(*tert*-butyldimethoxysilyloxy)butyryl]-*N'*-[4-(4,4'-dimethoxy-trityloxy)butyryl]-2-*O*-(*N*,*N*-diisopropylamino-2-cyanoethylphosphinyl)-1,3-diamino-2-propanol, were synthesized and used for the creation of plain and mixed oligonucleotide dendrimers. Our approach appears to be a convenient way to the synthesis of plain and mixed dendrimers that bear various functional moieties (including oligonucleotides) and possess a wide range of chemical and physical properties. The reagents suggested are easily available and compatible with automated solid phase phosphoramidite chemistry, which makes them potentially interesting for many biochemically-oriented laboratories.

Key words: branched phosphoramidite reagents; oligonucleotide dendrimers.

INTRODUCTION

Novel technologies have being developed on the interface of traditional disciplines of science and engineering. There is a need of new synthetic methods, which can fill a gap between the molecular scale and the nanoscale preparative techniques in bioorganic chemistry. We describe here a novel set of reagents and methods that allow the assembly of large branched structures (dendrimers) from simple but versatile building blocks.

First introduced in 1981 [1], the phosphoramidite approach has made the synthesis of oligonucleotides routine to all life-sciences laboratories thanks to its reliability and simplicity. Our range of branching synthons may expand the potential of phosphoramidite chemistry by creating two-dimensional and three-dimensional dendrimeric structures [2].

Branched oligonucleotides were found in nature [3] and were synthesized by using modified deoxy- or ribonucleosides as the branching points [4, 5]. Another approach to branched structures was based on the use of nonnucleoside forks [6]. Both synthetic schemes doubled the number of reactive groups after each condensation stage. More rapid growth might be achieved with more branched monomers.

Abbreviations: CPG, controlled pore glass; DMAP, *p*-dimethylaminopyridine; DMTr, 4,4'-dimethoxytrityl; EDIP, ethyldiisopropylamine; LCAA – CPG, long chain alkylamine controlled pore glass; Py, pyridine; TBAF, tetrabutylammonium fluoride; and TBDMSCl, *tert*-butyldimethylsilyl chloride.

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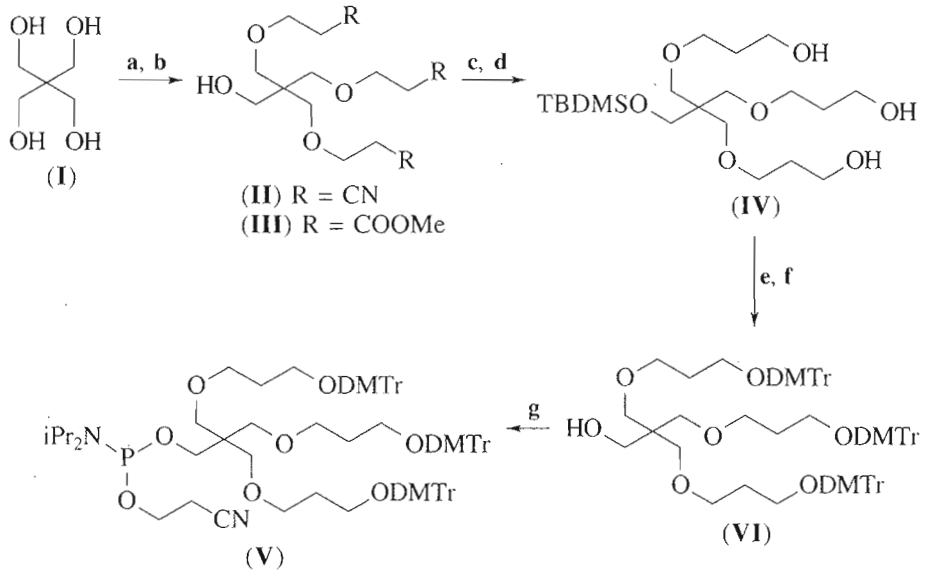
RESULTS AND DISCUSSION

We synthesized a new phosphoramidite trebling reagent (**V**) starting from pentaerythritol (**I**) (Scheme 1). Three of four hydroxyl groups of (**I**) were cyanoethylated by the reaction with acrylonitrile to give an intermediate trinitrile (**II**), which was converted to triester (**III**) by the Pinner reaction. The hydroxyl of triester (**III**) was protected by a treatment with *tert*-butyldimethylsilyl chloride, and the lithium borohydride reduction of the intermediate TBDMS-ether led to triol (**IV**). (**IV**) was tritylated with an excess of dimethoxytrityl chloride, and the resulting peretherified derivative was readily separated from the mono- and di-DMTr derivatives by column chromatography. Its desilylation by tetrabutylammonium fluoride in THF gave tris-2,2,2-[3-(4,4'-dimethoxytrityloxy)propyloxymethyl]ethanol (**VI**); MS (MALDI-TOF), *m/z*: 1241.64 $(M + Na)^+$ (calc. for $C_{77}H_{84}O_{13}$: *m/z* 1216.5); ^{1}H NMR ($CDCl_3$, δ, ppm): 7.5 – 6.7 (39 H, m, aromatic protons), 3.77 (18 H, s, 6 OCH_3), 3.45 (6 H, t, DMTrOC**H**$_2$), 3.3 (6 H, s, $(OCH_2)_3C$), 3.10 (6 H, t, CH_2O), 2.78 (1H, br. s, OH), 1.80 (6 H, q, $CH_2CH_2CH_2$), and 1.65 (2 H, br.s, C**H**$_2$OH). The target trebling phosphoramidite (**V**) was obtained by the treatment of (**VI**) with the corresponding phosphite; it exhibited MS (MALDI-TOF), *m/z*: 1413.22 $[M - H]^+$ (calc. for $C_{86}H_{101}N_2O_{14}P$, *m/z*: 1414.9); ^{31}P NMR (1 : 1 CH_3CN : CD_3CN, 80% H_3PO_4 as an internal standard, δ, ppm): 151.312; ^{1}H NMR ($CDCl_3$, δ, ppm): 7.45 – 6.7 (39 H, m, aromatic protons), 3.76 (18 H, s, 6 OCH_3), 3.70 (2 H, m, CH_2CH_2OP), 3.56 (2 H, m, CHN), 3.44 (6 H, t, DMTrOC**H**$_2$), 3.30 (6 H, s, $(OCH_2)_3C$), 3.08 (6 H, t, 3 CH_2O), 2.52 (2 H, t, CH_2CN), 1.79 (6 H, q,



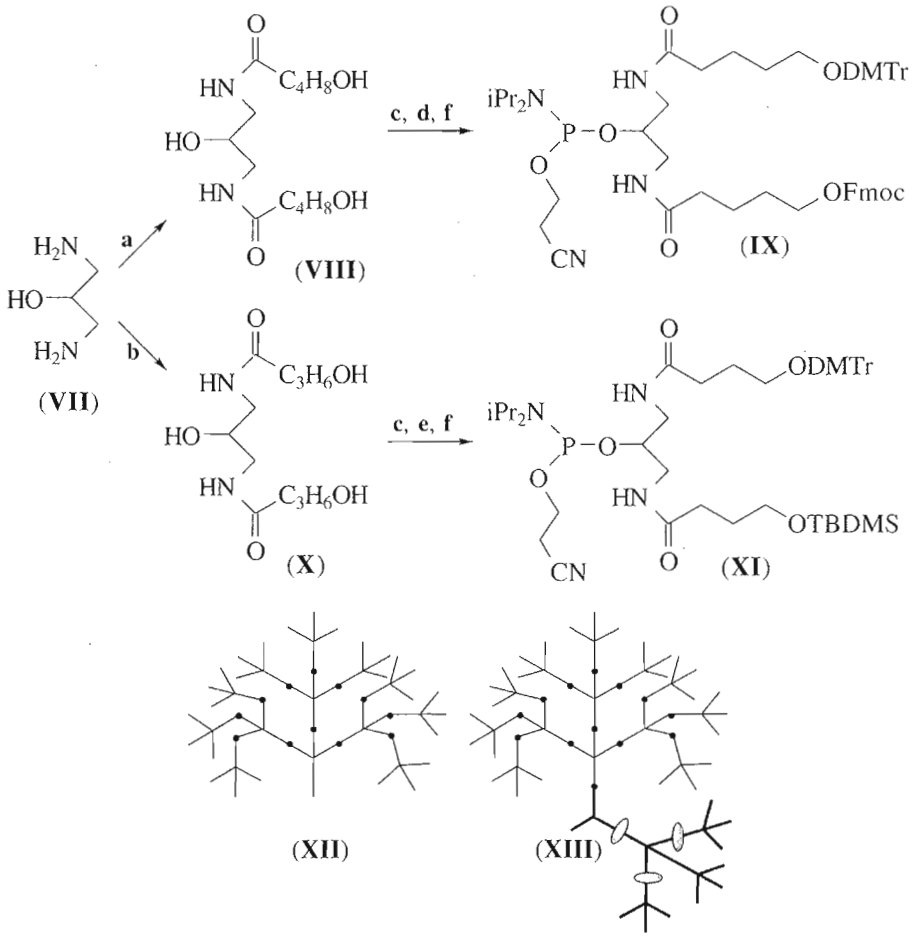
Scheme 1. Reagent: (**a**) acrylonitrile (3.1 equiv), aqueous NaOH, 50°C, 15 h 63.7%; (**b**) sat. HCl/MeOH, reflux, 2.5 h, 75%; (**c**) TBDMSCl (1.2 equiv)/Py, 10 h, room temperature, 98%; (**d**) $LiAlH_4$, dry THF, 3 h, 0°C to room temperature, 67%: (**e**) DMTrCl (4 equiv), Py/EDIP/DMAP, 7 h, room temperature, 58%; (**f**) 1 M TBAF/THF, 6 h, room temperature, 88%; (**g**) $iPr_2N(NCCH_2CH_2O)P$-Cl, EDIP, 0°C to room temperature, 1h, 75%.

$CH_2C\mathbf{H}_2CH_2$), 1.53 (2 H, m, CH_2OP), and 1.12 (2 H, t, *J* 6 Hz, $CHC\mathbf{H}_3$).

This reagent required no prolonged removal of DMTr protective groups in acidic conditions during oligonucleotide synthesis, as reported in [4]: at our synthetic scale (1 μmol, coupling time 2 min): about 80-s acidic treatment was sufficient for splitting off all its DMTr groups. The quantity of $DMTr^+$ cation released indicated that (**V**) gives stable trebling of the number of DMTr groups (yield >95%) for up to three successive couplings [see structure (**XII**), dendrimer of the third generation] when using a 500-Å DMTrT–LCAA–CPG support. Up to 4–5 couplings were carried out, when a 1000-Å CPG support was employed, which resulted in approximately 80–240 terminal hydroxyl groups. The elongation of the trebling branches with spacers like (O^1-DMTr,O^3-phosphoramidite)-propane-1,3-diol improved the yields of next coupling stages. The same effect can also be achieved with a spacer inserted between the first trebling unit and the solid support.

The coupling of (**V**) with 5'-OH-group of 5'-HO-T–LCAA–CPG and subsequent condensation of DMTrT phosphoramidite to three hydroxyl groups of this dimer led to 3'-$TpCH_2C(CH_2OCH_2CH_2CH_2pT\text{-}5')_3$, whose structure was confirmed by MALDI-TOF MS (positive ion reflector mode, delay time of 80 ns, 108 averaged scans), *m/z*: 1526.94 $[M + H]^+$, 1548.94 $[M + Na]^+$ (calc. for $C_{54}H_{81}O_{35}N_8P_4$, *m/z*: 1526.151). No trace of a product containing two terminal thymidine residues in place of three ones, as would result from an incomplete condensation, was detected. Oligonucleotide synthesis on top of dendrimeric structures like (**XII**) produced bunches of 3, 9, etc. oligonucleotide moieties (in general, 3n moieties, were n is the generation number) connected through either 3' or 5' ends depending on the type of nucleoside phosphoramidites used.

For the synthesis of mixed dendrimers bearing, for example, different oligonucleotide sequences, we designed branched monomers containing differently protected hydroxyl groups (Scheme 2). The choice of suitable protecting groups compatible with the oligonucleotide synthetic chemistry is limited. The previously described fork [7] containing DMTr- and *p*-methoxyphenyl protected OH-groups needs strongly oxidative conditions [Ce(IV) treatment] for deprotection and is unsuitable for labile synthons. We synthesized two new fork structures by the acylation of 1,3-diamino-2-propanol (**VII**) with δ-valerolactone and γ-butyrolactone to give (**VIII**) and (**X**), respectively. Two primary hydroxyls in (**VIII**) and those in the smaller compound (**X**) were protected with Fmoc and DMTr and with TBDMS and DMTr, respectively. The phosphitylation of their secondary hydroxyl groups led to the desired phosphoramidites: *N*-[5-(9-fluorenylmethoxy)valeroyl]-*N'*-[5-(4,4'-dimethoxytrityloxy)valeroyl]-2-*O*-(*N*,*N*-diisopropylamino-2-cyanoethoxyphosphinyl)-1,3-diamino-2-propanol (**IX**) {MS, MALDI-TOF, *m/z*: 1037.294 $[M + Na]^+$ (calc. for $C_{58}H_{71}N_4O_{10}P$, *m/z*: 1014); 1H NMR ($CDCl_3$, d, ppm): 7.77 – 6.72 (21 H, m, aromatic protons), 6.32 (1 H, br.t, NH), 6.29 (1 H, br.t, NH), 4.42 (2 H, d, CH_2 of Fmoc group), 4.22 (1 H, t, $C\mathbf{H}CH_2$ of Fmoc group), 3.78 (6 H, s, 2 OCH_3), 3.65 (2H, m, $CH_2C\mathbf{H}_2OP$),



Scheme 2. Reagents: (**a**) δ-valerolactone (4 equiv), DMAP (0.1 equiv), MeOH, reflux, 9 h, 90% recryst. from CH_2Cl_2; (**b**) synthesized as described in [8]; (**c**) DMTrCl (0.5 equiv) in Py, 0°C, 3h, 40–45%; (**d**) FmocCl (1.1 equiv) in Py, room temperature, 2 h, 70%; (**e**) TBDMSCl (1 equiv) in Py, room temperature, 4 h, 77%; (**f**) $(iPr_2N)_2POCH_2CH_2CN$, tetraline, 0°C to room temperature, 75–80%.

Dendritic structures (**XII**) and (**XIII**) were obtained dy using synthon (**V**) and a combination of (**V**) with (**IX**) or (**XI**).

3.55 (2H, m, CHN), 3.32 (4H, m, CH_2N), 3.12 (1H, m, POCH), 2.51 (2H, t, CH_2CN), 2.26 (4H, m, $C(O)CH_2$), 1.71 (12 H, m, $OCH_2CH_2CH_2$), and 1.1 (2H, t, *J* 6 Hz, $CHCH_3$)} and *N*-[4-(*tert*-butyldimethylsilyloxy)butyryl]-*N'*-[4-(4,4'-dimethoxytrityloxy)butyryl]-2-*O*-(*N*,*N*-diisopropylamino-2-cyanoethylphosphinyl)-1,3-diamino-2-propanol (**XI**) {^{1}H NMR ($CDCl_3$, δ, ppm): 7.5 – 6.75 (13 H, m, aromatic protons), 6.46 (1 H, br.t, NH), 6.35 (1 H, br.t, NH), 3.79 (6 H, s, 2 OCH_3), 3.67 (2 H, m, CH_2CH_2OP), 3.56 (2 H, m, CHN), 3.38 (4 H, m, CH_2N), 3.1 (1 H, m, CHOP), 2.52 (2 H, t, CH_2CN), 2.34 (4 H, m, $C(O)CH_2$), 1.89 (8 H, m, CH_2CH_2O), 1.11 (2H, t, *J* 6 Hz, $CHCH_3$), 0.9 (9 H, s, ^{t}Bu), 0.06 (6 H, s, Me); MS, MALDI-TOF, *m/z*: 902.156 [*M* + Na]$^+$, calc. for $C_{47}H_{71}N_4O_8PSi$, *m/z*: 879}. The condensation yields of (**IX**) and (**XI**) were high (>95% when increasing the condensation time, according to $DMTr^+$ assay).

Novel applications for dendrimers could be opened up if multiple terminal functionalities could be introduced onto the outer surface of dendrimers to give structures like (**XIII**). Such compounds can be readily synthesized on the basis of synthons (**IX**) and (**XI**), with the functionalities being introduced at any generation of dendrimer synthesis by means of (**V**). If different chemistries were then applied to two different branches after the removal of different protecting groups, the dendrimer with two different functionalities on the surface and possibly different internal links would result. The generation at which the differentiating synthon is introduced will determine the relative contribution of the two functionalities.

To synthesize mixed oligonucleotide dendrimers (**XIII**) for further use in solution, we made one condensation with (**IX**) on a CPG support and then used its DMTr-protected arm to build up a structure similar to (**XII**). After final detritylation and exhaustive capping (2 min), the second primary hydroxyl group was deprotected as described in [9]. Synthesis was then carried out as usual to generate the second half of (**XIII**), fol-

lowed by a routine deprotection. TBDMS-containing synthons are not compatible with CPG supports as TBAF destroys the glass. Nevertheless, other solid supports such as Tentagel or aminated polyproplylene [10] can be used with this reagent.

The characterization of our new compounds, especially those of higher generations, is not easy and will be a subject of separate publications. Lower generations (up to 2) can be characterized initially by measuring the released DMTr-cation, and, next, by using MALDI-TOF mass spectrometry. We analyzed the higher generation compounds by using both the $DMTr^+$ released quantity and the mobility shift in PAGE and HPLC. The radioactive 5'-labeling of oligonucleotide branches assembled into the dendritic structure through their 3'-ends was also used to estimate the number of end groups, which up to 3th–4th generation corresponded well to the expected value. Oligonucleotides of different generations synthesized using our reagents are currently under study as polylabeled probes in oligonucleotide array technology. The properties of plain (**XII**) and mixed (**XIII**) dendrimeric oligonucleotides are under investigation.

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