Pyrolysis Mass Spectrometry of Protected Phosphotriester Oligodeoxyribonucleotides

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Pyrolysis mass spectrometry is shown to be a useful tool in the analysis of the protected intermediate deoxynucleotides synthesized by the phosphotriester approach. Characteristic ions of the protected nucleic acid bases and terminal substituents appear in the mass fragmentation pattern which allows a rapid control of the attachment of the polynucleotide block in the chemical synthesis.

Under pyrolytic conditions DNA has been subjected to mass spectrometry analysis [1–3]. Cleavage of the phosphodiester bonds linking the nucleotide residues followed by subsequent fragmentation and rearrangement result in ions characteristic of the nucleobases and deoxyribose fragments. The method has been extended to dinucleotides protected on the amino and hydroxyl functions which have also an internucleotidic bond of the phosphodiester type [4].

Independently [5], we have studied the pyrolysis mass spectrometry fragmentation of fully protected oligonucleotides obtained as key intermediates in the phosphotriester approach of gene synthesis. We wish to report herein the results obtained with these phosphotriester products. The method enables the direct analysis of very small amounts of material without prior chemical derivatization or enzymatic treatment.

Experimental

Synthesis of the fully protected phosphotriester oligonucleotides

The hexanucleotide d(MeoTr – bzA(CIPh) – bzA(CIPh) – acG(CIPh) – T(CIPh) – Tac) was prepared by condensation of d(MeoTr – bzA(CIPh) – acG(CIPh) – T(CIPh) – Tac) by the general procedure previously described for phosphotriester oligonucleotide synthesis [6–17].

The protected trinucleotides were obtained by the method recently described by Cashion et al. [6]. d(MeOTr – bzC) was phosphorylated and then condensed with dThd to give d(MeOTr bzC(CIPh) – T). The same procedure was used to prepare d(MeOTr – bzA(CIPh) – T(CIPh) – T), d(MeOTr – bzC(CIPh) – T) was phosphorylated by an excess of imidazolyl-triazolyl-p-chlorophenylphosphate and the reacted material condensed with dThd. Acetylation and detritylation were performed as usual [10]. A similar method was used to obtain d(MeOTr – bzA(CIPh) – bzA(CIPh) – acG), which was subsequently phosphorylated and cyanoethylated as described previously [10, 11].

The Cashion procedure [6] is simple and efficient at least for the synthesis of di- and trinucleotides. For higher oligonucleotides the yields tend to decrease. Under the set of conditions used by the authors (for example d(MeOTr – bzC) 1 equiv., p-ClPdoClpa 1.2 equiv., triazole 4 equiv., triethylamine 4 equiv., CH₃-imidazole 16 equiv., dThd 3 equiv.) it appears that 3′ → 3′ condensation, as shown by 31P NMR (unpublished work), is very small and that the corresponding products are eliminated during the chromatographic purification steps.

The products were separated by preparative high pressure liquid chromatography using silica gel fine particles thus improving the practical yields of final products compared to those obtained by thin layer silica gel chromatography. Formation of highly coloured side products, which proved troublesome in the separation step, has since been reduced by replacing pyridine by dioxane or tetrahydrofuran as the condensation solvent. However, traces of pyridine seem to be useful as catalyst.

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Characterization of the product

The hexanucleotide was detritylated by a mixture of benzene sulfonic acid in chloroform-methanol [10]. The phosphate and amino protecting groups were eliminated by treatment with ammonia [10]. The deprotected hexanucleotide was purified by PARTISIL 10 SAX High Performance Liquid Chromatography [18]. The sequence was verified by the method of van Boom using phosphodiesterase [19].

Mass Spectrometry Analysis

Mass spectra recording

The mass spectrometer, AEI-Kratos, was connected to a computer system (Sysmas-Telemechanique). Samples (1–2 μg) were placed at the end of a glass tube and introduced into the ion-source, heated at about 180 °C, working in electron impact at 70 eV.

At fixed intervals a mass spectrum was scanned and memorised. If necessary, high resolution spectra were scanned with perfluorokerosene to give the atomic composition of each ion. Reproducibility of spectra was controlled.

Substituents

After ionization, the 5'-terminal substituent paramethoxytrityl radical is liberated thermally and appears at m/e 273 with relative fragments (m/e 165 by MeOC₆H₅ elimination). The trityl radical may be hydrogenated in the ion source or in the sample and then appears at m/e 274 (MeOTr H)+ and m/e 197 (phenyl elimination). The molecule MeOTr OH (molecular ion m/e 290 and main fragment at m/e 213, phenyl elimination) is of a lower intensity.

Ions corresponding to MeOTr OH with one or two deoxyribose residues appeared at:

- m/e 370 for [MeOTr OH + 80]⁺,
- m/e 450 for [MeOTr OH + 2 × 80]⁺.

When the 3'-terminal phosphate was cyanoethylated the acrylonitrile molecular ion at m/e 53 was observed: CH₂=CH-CN.

The molecular ion derived from product 9 (Fig. 1) is also detected and is of relatively high intensity when the nucleotide is unsubstituted in the 5' position.

When the 3'-hydroxyl terminus is acetylated acetic acid is liberated thermally and observed at m/e 60 and 43.

Application to the control of synthesis

Comparison of the fragmentation pattern of the hexanucleotide and of the two trinucleotides used in its synthesis is shown in Fig. 2. In this way, it is possible to follow the progression of the synthesis.

However, it is important to note that the plot of intensity against time for a given ion is dependent on the nature of the ion and of the oligonucleotide from which it is derived. Fig. 3 shows the intensity of the main molecular ions against time during the pyrolysis of the hexanucleotide.

Characteristic ions

Pyrolysis of the fully protected deoxyribonucleotides in the mass spectrometer ion source gave rise to volatile products which were identified by their mass fragmentation pattern (Fig. 1).

Nucleobases

Benzoyladenine (m/e 239) gave m/e 211 and 210 by carbon monoxide and hydrogen elimination and m/e 105 (C₆H₅CO)+.

Acetylguanine (m/e 193) gave m/e 151, deprotected guanine, by ketene elimination.
Fig. 2. Comparison of mass spectra. The hexanucleotide is obtained by condensation of the two trinucleotides. The characteristic ions are indicated by A: adenine, G: guanine, C: cytosine, T: thymine, MTr: methoxytrityl.
Benzoylcytosine (m/e 215) gave m/e 214 (H elimination) m/e 187, 186 by carbon monoxide and hydrogen elimination, m/e 138 by phenyl elimination and m/e 105 (C₆H₅CO)⁺.

Thymine (m/e 126) gave m/e 83 by cyanic acid elimination.

In addition each nucleobase was observed associated with one or two deoxyribose residues, these ions being obtained probably by monochlorophenylphosphoric acid 6 elimination. These molecules are designated by [BH + 80] and [BH + 2 x 80], the latter being of low abundance.

Their molecular ions are:

- m/e 319 and 399 for benzoyladenine,
- m/e 295 and 375 for benzoylcytosine,
- m/e 273 and 353 for acetylguanine,
- m/e 206 for thymine (m/e 286 was not observed).

Parachlorophenylphosphoric acid was identified though its molecular ion (m/e 208, 210) is weak, being of low stability (its main fragment ion, parachlorophenol, (m/e 128, 130) is derived mainly from pyrolysis molecules).

Thymine, whose simple composite ions (BH+80...) were of low intensity, gave rise to an ion of higher m/e value 7 (C₁₅H₁₅N₂O₂PCl), which is a (BH + 80) ion with a p-chlorophenylphosphoric acid m/e 414 attached. It appears at the end of pyrolysis.

The methoxytrityl ions can be detected just after sample introduction into the ion source and are the first ions to be liberated. The chlorophenyl ions attain a maximum of intensity later in the pyrolysis.

Nucleobase ions also show different rates of evolution. Thymine ions are relatively low in reaching a maximum in intensity and the ions [Thy + 80]+ are of lower relative abundance than the other [BH + 80]+ ions.

Conclusion

Due to the high molecular weights and the complexity of protected phosphotriester oligonucleotides, which are isolated in small quantities as a mixture of diastereoisomers, the current available spectroscopic methods, NMR, IR, conventional mass spectrometry, are unsuitable for their characterization. Usually they are neglected and the protected phosphotriester oligonucleotides are not characterized. The structures of the resulting oligo-
nucleotides are thus determined after deprotection by the "fingerprint" method [20] or by the system of Maxam and Gilbert [21]. The structures of the protected oligonucleotides are inferred from the results obtained after their deprotection. However, it is useful to have a method to check rapidly the nature of the protected oligonucleotide after the condensation step in the phosphotriester approach.

The method has proved equally useful with oligonucleotides having the more currently accepted protecting groups: dimethoxytrityl, anisoyl and isobutyryl.

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