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A STUDY ON SPIN-LABELLED OLIGONUCLEOTIDE SYNTHESIS AND ITS ELECTRON SPIN RESONANCE BEHAVIOR IN SOLUTION

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An oligonucleotide spin-labelled with 4-amino-2,2,6,6-tetramethylpiperidine-N-oxyl (4-amino-TEMPO) at the internucleotide bond (d-Tp(L)TpTpTpT) prepared by oxidation of the pentanucleotide containing the H-phosphonate diester (d-Tp(H)TpTpTpT) in the presence of 4-amino-TEMPO, was separated and identified by high-performance, reverse-phase liquid chromatography combined with detection by electron spin resonance spectroscopy. This spin-labelled oligonucleotide produced a triplet with the slightly broadened $M_I = -1$ ESR component, while a triplet with almost equal intensities was obtained from the spin-label. The $M_I = -1$ component from the labelled oligonucleotide was further broadened in the presence of poly(A) which forms a complementary double strand with this molecule.

KEY WORDS: Oligonucleotide, spin-label, ESR, hybridization.

INTRODUCTION

DNA probe[1] diagnoses for detecting genetic defects have become one of the most important research targets in medicine. Recently several kinds of non-radioactive labelled DNA probes, for instance fluorescent-labelled ones,[2] which are safe and stable compared to conventional radioactive DNA probes, have been invented. Upon usage of such probes, very low quantities of DNAs can be detected. In order to detect such quantities of DNAs, as an alternative method, we have tried to use electron spin resonance (ESR) detection systems, namely "spin-labelled DNA probes.[3,4]". Since only spin-labelled DNA is detected by ESR and the ESR signals can be accumulated by desital multiplication, there will be high sensitivity in this method. Other advantages of this method are safety, ease of handling and storage, and so on. In this preliminary study, therefore, the purpose is focused on the preparation of a spin-labelled oligonucleotide and the exploration of the characteristics of its ESR signals in solution. The sample chosen here is spin-labelled pentanucleotide (d-Tp(L)TpTpTpT).

Recently the nucleoside H-phosphonate method[5,6] was shown to be useful in the chemical synthesis of a single-stranded DNA. In this method, the synthesis is proceeded with polynucleoside H-phosphonate diester intermediates. Oxidization of the H-phosphonate intermediates in the presence of amines leads to the corresponding phosphoramidates.[7] We took this method for the synthesis of d-Tp(L)TpTpTpT.

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Although this synthesis can be used to prepare spin-labelled DNAs, many by-products are also formed in such quantities that they can not be used as DNA probes directly. Since we have already demonstrated that high-performance reverse-phase liquid chromatography (RPLC) is effective in the purification of chemically synthesized DNAs,[8,9] and useful for the separation of the targeted products, RPLC was utilized here. Since only the targeted product must have the spin-label and can be detected by ESR, in the present study the spin-labelled product was separated and identified by the combination of RPLC separation and ESR detection[10] (RPLC-ESR system).

SYNTHESIS OF SPIN-LABELED OLIGONUCLEOTIDE

5′-O-dimethoxytritylthymidine (DMTr–T) and silica gel on which DMTr–T was bound (DMTr–T–Si) were prepared by reported procedures.[11,12] Tetrathymidylate bound on silica gel (d-TpTpTpT–Si) and 5′-O-dimethoxytrityl-3′-thymidine H-phosphonate were prepared according to the published procedures.[5]

The synthesis of d-Tp(L)TpTpTpT was carried out in a gas-tight syringe,[13] into which glass wool was inserted so that the silica gel, utilized as a reaction bed, was

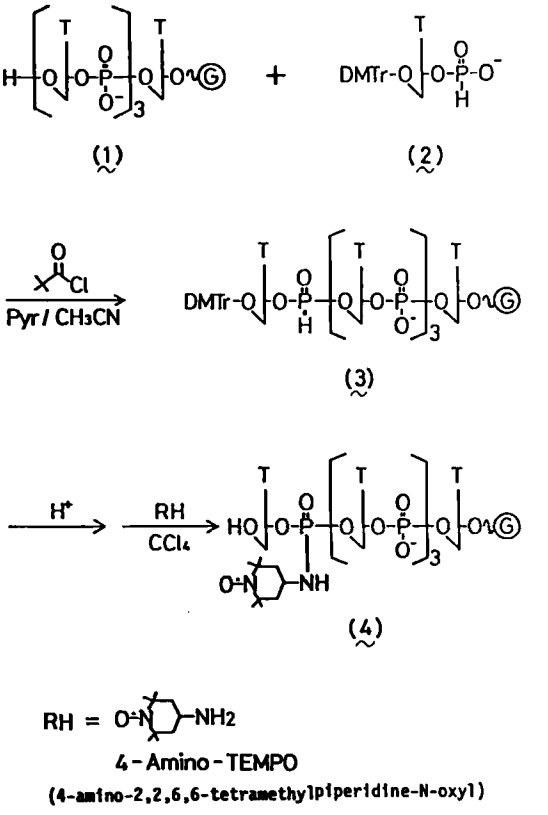

FIGURE 1 Schematic illustration of the synthesis of d-Tp(L)TpTpTpT.

stored in the syringe. The procedures, illustrated in Figure 1, were followed. To d-TpTpTpT–Si (1), washed with CH_3CN, 5′-O-dimethoxytrityl-3′thymidine H-phosphonate (2) and pivaloyl chloride were added and the system was allowed to stand for 5 min[5] to yield (3). The yield of this reaction was determined, by monitoring trityl cation released from the resultant support by 2.5% $CHCl_2COOH$ in CH_2Cl_2, to be 70%. After the removal of the dimethoxytrityl group and CH_3CN washing, thus prepared d-Tp(H)TpTpTpT–Si was treated for 30 min with 4-amino-TEMPO (40-fold equivalent to DMTr-T which was originally bound on silica gel) in CCl_4, producing (4). After CH_3CN washing, the resultant products were removed from the solid support by treating with conc. NH_4OH for 6 hr. Each synthetic step was carried out at ambient temperature. Subsequently the solvents and remaining additives were removed by evaporation at ca. 30°C under reduced pressure.

The products mixture was dissolved in water and analyzed by RPLC with UV-monitoring under the conditions mentioned in EXPERIMENTAL SECTION. As represented in Figure 2a, many peaks appeared in the chromatograms, indicating the concomitant by-products in the sample. The peak due to the targeted product, d-Tp(L)TpTpTpT, could not be recognized because of the high quantities of impurities. In order to distinguish the peak due to the targeted compound, ESR detection was applied, since only the target product is expected to have the spin-label.

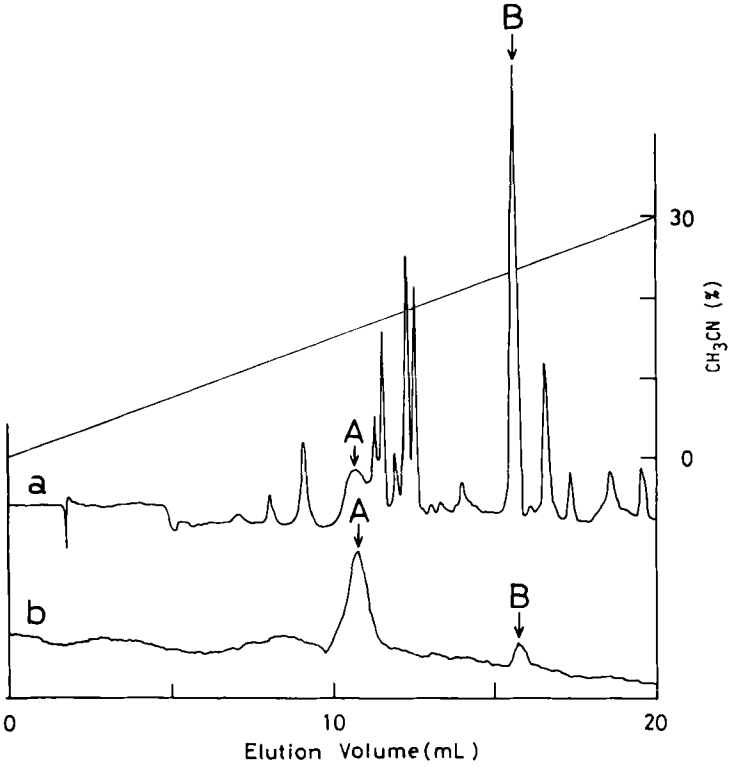

FIGURE 2 Chromatograms obtained from the synthetic mixture of d-Tp(L)TpTpTpT by (a) UV and (b) ESR detection. For chromatographic conditions and ESR settings, see EXPERIMENTAL SECTION.

Shown in Figure 2b is a chromatogram obtained from the same sample by the ESR monitoring. In the ESR monitored chromatogram, two peaks indicated by A and B were obtained. For the characterization of the peaks, a solution of 4-amino-TEMPO was loaded on the same system and the peak A was found to be due to this compound. This result implies that 4-amino-TEMPO was not washed out completely by the CH_3CN washing after the spin-labelling reaction, which has been reported to be effective in the conventional synthesis of DNAs.[5,6] Consequently peak B was determined to be due to d-Tp(L)TpTpTpT. Although it has been reported that the phosphoramidate synthesis via H-phosphonate gives rise to the formation of equal amounts of stereoisomers,[7] the formation of 4-amino-TEMPO phosphoramidate stereoisomers could not be observed in the present study.

Since we have already developed an RPLC column which is suitable for the purification of chemically synthesized large DNAs such as those consisting of 50

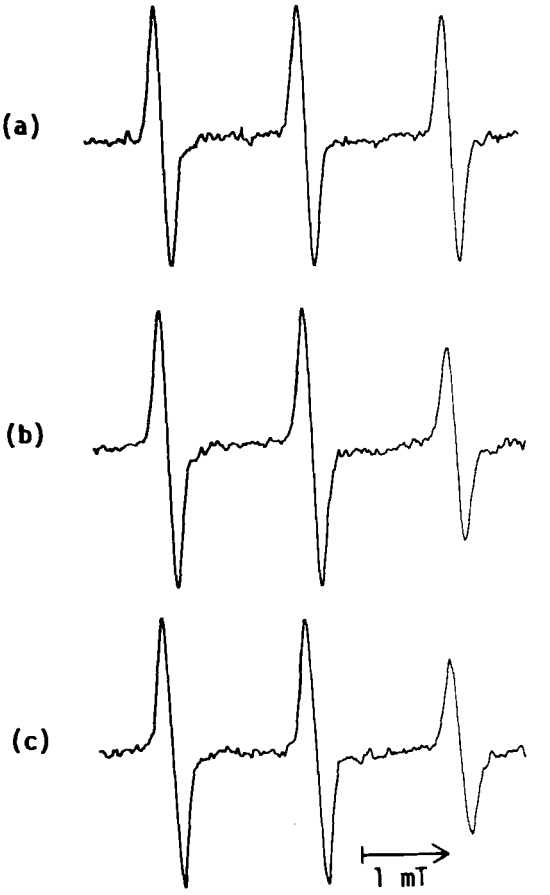

FIGURE 3 ESR spectra obtained from (a) 4-amino-TEMPO, (b) d-Tp(L)TpTpTpT, and (c) d-Tp(L)TpTpTpT in the presence of poly(A) (50-fold equivalent to d-Tp(L)TpTpTpT) and 0.9 M NaCl, in 0.1 M TEAB buffer (pH 7.5) at ambient temperature. ESR settings: microwave power, 10 mW; modulation amplitude, 0.04 mT; gain, X10000; time constant, 10 sec; scan time, 128 min.

bases,[8,9] this RPLC-ESR system will be useful for the preparation of spin-labelled DNAs even larger than the sample studied here.

ESR STUDIES OF SPIN-LABELLED OLIGONUCLEOTIDES

In order to see how the spin-labelled oligonucleotide behaves in solution, the following ESR measurements were performed in a conventional manner. Represented in Figure 3a is an ESR spectrum obtained from 4-amino-TEMPO in 0.1 M triethylammonium bicarbonate (TEAB) buffer (pH 7.5). This spectrum is composed of a triplet with equal intensities (a_N = 1.71 mT). Because of a fairly large hyperfine splitting constant (a_H = 0.04 mT) of the methyl groups located at the axial positions (C2 and C6),[14] this triplet is originally broad, even though the molecule is freely rotating. d-Tp(L)TpTpTpT was measured in TEAB buffer (pH 7.5) by ESR, producing a spectrum depicted in Figure 3b (a_N = 1.71 mT). The triplet of this spectrum is slightly broadened compared to that of 4-amino-TEMPO. In particular, the $M_I = -1$ component is fairly broadened. This implies that there is steric hindrance due to the main chain preventing free rotation of the spin-labelling group.

Further measurement was carried out to see whether the spin-labeled oligonucleotide makes a duplex with poly(A). When d-Tp(L)TpTpTpT was dissolved in 0.1 M TEAB buffer (pH 7.5) containing poly(A) (50-fold equivalent to d-Tp(L)TpTpTpT) and 0.9 M NaCl, the $M_I = -1$ component of a triplet (a_N = 1.71 mT shown in Figure 3c was further broadened, indicating the change of the circumstances of d-Tp(L)TpTpTpT, presumably due to the duplex formation.

In summary, a pentanucleotide spin-labelled with 4-amino-TEMPO at the internucleotide bond, d-Tp(L)TpTpTpT, could be obtained by oxidation of d-Tp(H)TpTpTpT in the presence of 4-amino-TEMPO and by subsequent RPLC separation aided by ESR dectection. This implies that both the spin-label and the phosphoramidate linkage of the product were stable under the oxidizing conditions and those necessary for the removal of the N-protecting groups. The spin-labelled oligonucleotide produced broadened ESR line of the $M_I = -1$ component compared to that of the spin-label and the line was further broadened in the presence of poly(A). These results indicate that the spin-labelled DNA probe method is feasible for practical usage.

EXPERIMENTAL SECTION

Thymidine and poly (A) were purchased from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan) and 4-amino-2,2,6,6-tetramethylpiperidine-N-oxyl (4-amino-TEMPO) was obtained from Aldrich Chemical Co. (Milwaukee, Wis., U.S.A.). Pivaloyl chloride and other reagents were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Carbon tetrachloride was dried over molecular sieves 4A. Pyridine was refluxed over CaH_2 for 6 hr and distilled over molecular sieves 4A. Acetonitrile and dichloromethane used were also anhydrous. Silica gel (Wakogel C-200) used as a support in the DNA synthesis was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

A high-performance liquid chromatograph used was CCPM (Tosoh, Tokyo, Japan) equipped with a UV-flow monitor (UV-8000, Tosoh). The chromatography conditions were as follows: column, TSKgel ODS-120T (4.6 mm i.d. and 15 cm in

length, Tosoh); eluent, (high) 30% CH_3CN containing 0.1 M CH_3COONH_4 and (low) 0.1 M CH_3COONH_4; gradient, linear, from low to high for 40 min; flow rate, 0.5 mL/min; detection, 254 nm; temperature, ca. 25°C.

The chromatograph was also combined with an ESR spectrometer (Model PE-3X, JEOL, Tokyo, Japan), operated at 100 kHz modulation in the X-band. A quartz ESR flow cell (0.8 mm i.d. and 18 cm long), which was fixed in the ESR sample cavity, was connected to the exit of the UV-flow monitor with teflon tubing. The ESR settings utilized for the detection of free radicals in the effluent were as follows: microwave power, 10 mW; magnetic field, fixed at 334.3 mT; modulation amplitude, 0.63 mT.

Conventional ESR measurements were carried out on a FE2XG spectrometer (X-band, 100 kHz field modulation, JEOL) using an aqueous flat cell at ambient temperature.

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