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Chemical Synthesis of Deoxyribonucleotides Containing Deoxyadenosine at the 3′-End on a Polystyrene Polymer Support

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Deoxyribonucleotides containing 2′-deoxyadenosine at the 3′-terminal, *i.e.*, a heptadeoxyribonucleotide, d-GATTCTA, two pentadecadeoxyribonucleotides, d-A_{15}, d-AATGAGAAGCAACGA and a nonadecadeoxyribonucleotide, d-AGAATCTCGTTGCTTCTCA, were synthesized on a polystyrene polymer support by using the phosphotriester method. Dimer blocks (**3**) were coupled for elongation in the 5′-direction by using 1-mesitylenesulfonyl-3-nitro-1*H*-1,2,4-triazole as the activating reagent. A 3% trichloroacetic acid solution in dichloromethane was used for the detritylation process without causing any detectable depurination. 5′-*O*-Phosphorylated heptanucleotide and pentadecanucleotide were successfully joined by using deoxyribonucleic acid ligase in the presence of a template.

Keywords——solid-phase synthesis; phosphotriester method; deoxyadenosine; DNA ligase

Deoxyribooligonucleotides are indispensable tools in biological studies. For example, they are used as substrates[1,2] for deoxyribonucleic acid (DNA) ligase, as primers[3,4] for reverse transcriptase and DNA polymerase, and as probes[5,6] for hybridization experiments. Occasionally they are also used as tools for *in vitro* mutagenesis studies.[7] The necessary short-chain deoxyribonucleotides can be rapidly synthesized on a polymer support.[8-11] However, some problems still remain to be solved, especially in relation to the acid-catalyzed removal of the dimethoxytrityl group. Namely, the synthesis of deoxyribooligonucleotides containing deoxyadenosine at the 3′-end has been reported[12,13] to be difficult due to the instability of N^6-benzoyldeoxyadenosine to acid treatment. Benzenesulfonic acid and trichloroacetic acid cause the dupurination of N^6-benzoyldeoxyadenosine in solution.[14] Only zinc bromide treatment is known to give satisfactory results.[15,16] However, on a polymer support, it takes more than 30 min to remove the dimethoxytrityl group completely from the 5′-hydroxyl group with zinc bromide.[17] Some improvements have been reported to overcome this problem. The phthaloyl protecting group has been used in place of the benzoyl group by Kume *et al.*[18] N^6-Dialkylaminomethylenedeoxyadenosines were introduced by Froehler *et al.*[19] Hydrolysis of the dimethoxytrityl group with benzenesulfonic acid or trichloroacetic acid in various solvent systems has also been examined.[17] In this paper, we report that 3% trichloroacetic acid in dichloromethane was effectively used for the removal of the 5′-*O*-dimethoxytrityl group in such unstable systems, and by using this technique, the synthesis of a hepta-, two pentadeca- and a nonadecadeoxyribonucleotides containing deoxyadenosine at the 3′-end was achieved.

Results and Discussion

The synthetic scheme for the hepta-, pentadeca- and nonadecadeoxyribonucleotides is shown in Fig. 1. N^6-Benzoyl-5′-*O*-dimethoxytrityldeoxyadenosine bound to polystyrene resin (**1**) was prepared according to the known procedure.[11] Removal of the trityl group from **1**

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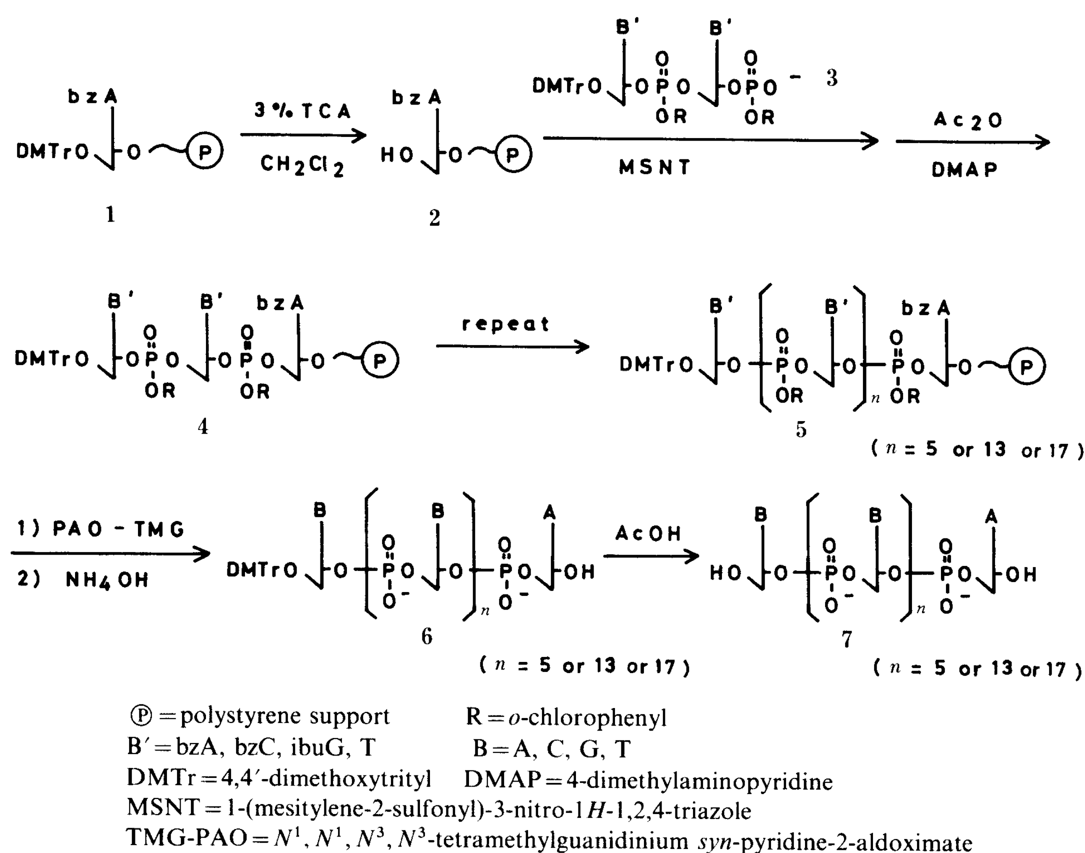

Fig. 1. Reaction Scheme for the Synthesis of Hepta-, Pentadeca- and Nonadecadeoxyribonucleotides

using 3% trichloroacetic acid was examined and deprotection was found to proceed without any detectable depurination when 3% trichloroacetic acid in dichloromethane was used and contact of the acid with the resin was limited to 2 min. Dimer blocks were used for the coupling to decrease the total time required for detritylation. Initially, we examined the synthesis of a heptadeoxyribonucleotide, d-GATTCTA. The triethylammonium salt of the protected dimer (**3**) (4 eq) was coupled with the resin (**2**) in the presence of 1-mesitylenesulfonyl-3-nitro-1*H*-1,2,4-triazole (MSNT).[20] Unreacted nucleosides were capped by the addition of acetic anhydride in pyridine containing 4-dimethylaminopyridine (DMAP). The reaction was continued for 2 min, although the acetylation was reported to complete within 30 s.[17,21] The operations necessary for one cycle of the coupling reaction of dinucleotides are shown in Table I. The total time required for each cycle was 1 h. The cycle was repeated using the appropriate dimer until the heptamer was obtained. The polymer-supported heptamer (**5**, $n=5$) thus prepared was treated with N^1,N^1,N^3,N^3-tetramethylguanidinium *syn*-pyridine-2-aldoximate (TMG-PAO) and concentrated ammonia to remove the phosphate and base protecting groups and to cleave the product from the resin. The product, which still possessed a dimethoxytrityl group, was separated by reversed-phase (C_{18} silica gel) column chromatography. The major peak contained the corresponding 5′-*O*-dimethoxytritylated heptamer (**6**, $n=5$). After aqueous acetic acid treatment for the removal of the dimethoxytrityl group, the desired heptamer, d-GATTCTA (**7**, $n=5$), was analyzed by reversed-phase (C_{18} silica gel) high pressure liquid chromatography (HPLC) (Fig. 2a). Next, we examined the effect of the acid treatment on the depurination of N^6-benzoyldeoxyadenosine. We again synthesized the heptamer with the same sequence according to the Table I except for the detritylation step. The acid treatment was prolonged to 20 min, ex-

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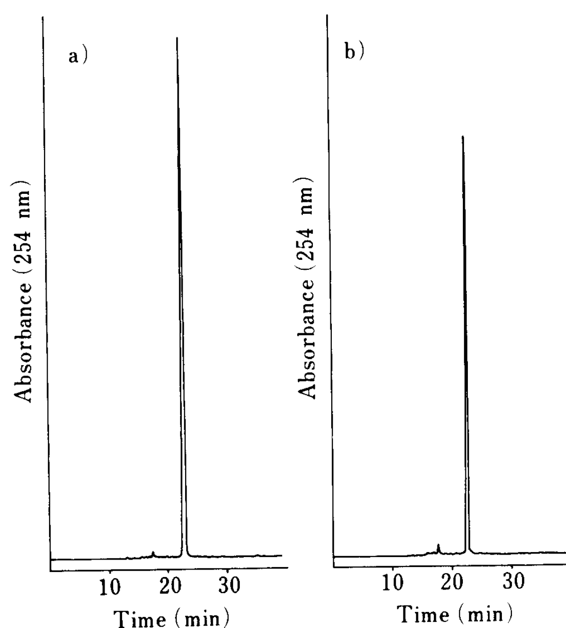

Fig. 2. HPLC Analysis of d-GATTCTA after a) Short or b) Long Acid Treatment on a Column of SSC PAK ODS-272

Elution was performed with a linear gradient of acetonitrile (10—20%) in 0.1 M TEAA (pH 7) for 1 h at a flow rate of 1 ml per min.

TABLE I. Schedule for One Cycle of Synthesis

Operation	Solution	Volume
1) Wash	CH_2Cl_2	1 ml (5 times)
2) Detritylation	3% Cl_3CCOOH in CH_2Cl_2	1 ml for 30 s (4 times)
3) Wash	Tetrahydrofuran	1 ml (3 times)
4) Wash	Pyridine	1 ml (2 times)
5) Coevaporation	Dimer (**3**) + pyridine	0.2 ml
6) Condensation	MSNT + pyridine	0.5 ml (45 min)
7) Wash	Pyridine	1 ml
8) Capping	1 M Ac_2O, 50 mM DMAP in pyridine	1 ml (2 min)
9) Wash	Pyridine	1 ml (2 times)

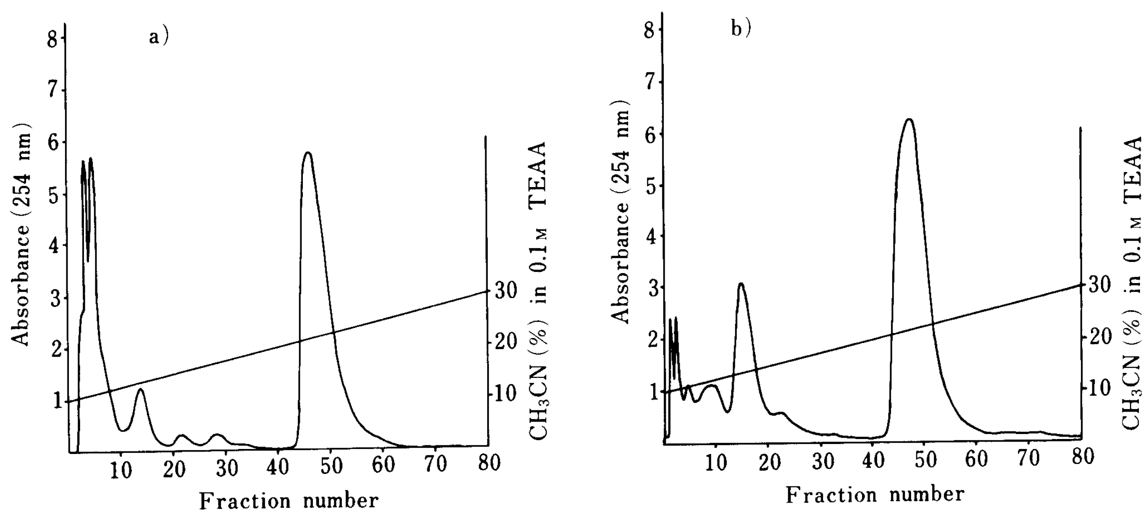

Fig. 3. Isolation of a) d-(DMTr)A_{15} and b) d-(DMTr)AGAATCTCGTTG-CTTCTCA on a Reversed Phase (C_{18}) Silica Gel Column

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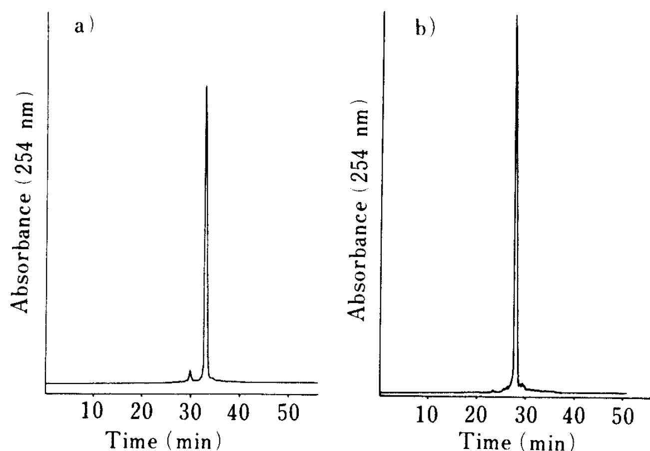

Fig. 4. HPLC Analysis of a) d-A_{15} and b) d-(DMTr)AGAATCTCGTTGCTTCTCA on a Reversed Phase (C_{18}) Silica Gel Column

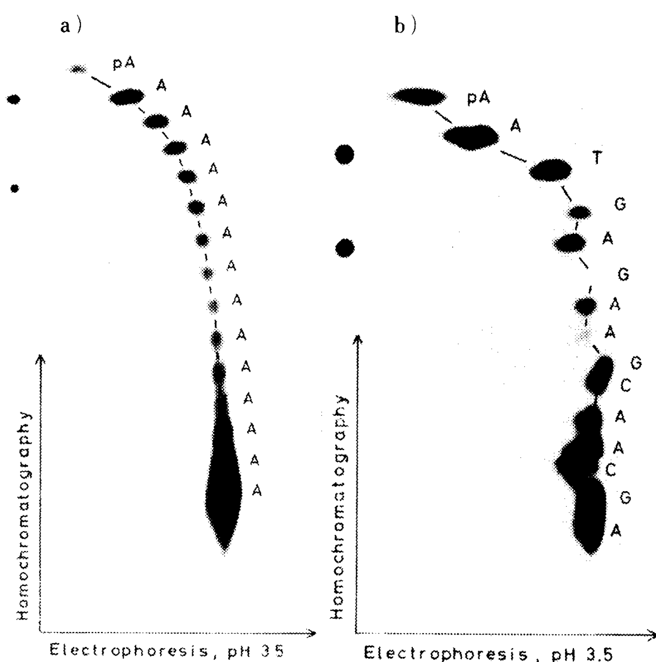

Fig. 5. Two-Dimensional Sequence Analysis of a) d-A_{15} and b) d-AATGAGAAGCAACGA

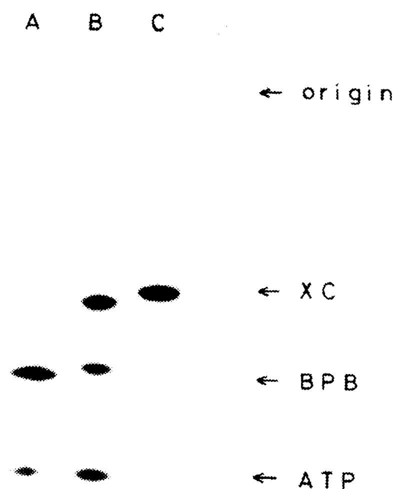

Fig. 6. Autoradiograph of a 20% Polyacrylamide Gel Electrophoregram of T_4-DNA Ligase Reaction Mixture

Lane A, [5′-^{32}P]d-GATTCTA; lane B, T_4-DNA ligase reaction mixture; lane C, 25-mer as a size marker; BPB, bromophenol blue marker; XC, xylene cyanol marker.

cept for the first acid treatment (2 min). Thus, N^6-benzoyldeoxyadenosine bound to the support was exposed to the acid for 42 min in total. After synthesis and deprotection as usual, the partially protected or non-protected heptamer was analyzed by HPLC (Fig. 2b). However, no difference between the two chromatograms was observed. The isolated heptamer was identified by enzyme degradation and mobility shift analysis.[22,23]

These results encouraged us to synthesize two pentadecadeoxyribonucleotides, d-A_{15} and d-AATGAGAAGCAACGA, and a nonadecadeoxyribonucleotide, d-AGAATCTCGTTGCTTCTCA, as summarized in Fig. 1 and Table I. The overall yields after six or eight couplings were 42% for d-A_{15}, 52% for d-AATGAGAAGCAACGA and 66% for d-AGAATCTCGTTGCTTCTCA as calculated from the released trityl cation. After seven or nine couplings with the appropriate dimer, the loaded polystyrene was treated with TMG-

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PAO and then with concentrated ammonia. The crude dimethoxytritylated pentadecamers (**6**, $n=13$) or nonadecamer (**6**, $n=17$) thus obtained were initially subjected to reversed phase (C_{18} silica gel) column chromatography (Fig. 3). The major peak corresponds to penta- or nonadecamer. After the dimethoxytrityl group has been removed with aqueous acetic acid, the completely deblocked penta- or nonadecamers (**7**, $n=13$ or 17) were purified by HPLC on a reversed phase (C_{18}) silica gel (Fig. 4). In order to confirm their sequences, purified penta- or nonadecamers were phosphorylated by using [γ-^{32}P] adenosine triphosphate (ATP) and T_4-polynucleotide kinase. These 5′-^{32}P-labeled pentadecamers were partially degraded with snake venom phosphodiesterase or nuclease P_1. The samples were analyzed by two-dimensional chromatography.[22,23] The result is shown in Fig. 5 in the case of pentadecamers. The sequence analysis gave satisfactory results.

Finally, in order to confirm that the d-AATGAGAAGCAACGA synthesized above is biochemically active, d-AATGAGAAGCAACGA and 5′-^{32}P d-GATTCTA were joined in the presence of T_4-DNA ligase and d-AGAATCTCGTTGCTTCTCA as a template. The result is shown in Fig. 6. The autoradiogram showed that the 22-mer (lane B) was formed from the pentadecamer and heptamer. Therefore, the d-AATGAGAAGCAACGA was found to be a good substrate for the enzyme reaction using T_4-DNA ligase.

In conclusion, we encountered no problems in the syntheses of penta- or nonadecadeoxyribonucleotides containing deoxyadenosine at the 3′-end by using 3% trichloroacetic acid in dichloromethane for the detritylation step. The pentadecamers thus synthesized were found to be biochemically active in every respect tested. Accordingly, the detritylation procedure using 3% trichloroacetic acid in dichloromethane was proved to be available for the synthesis of all types of deoxyribooligonucleotides, provided that a contact of the acid with the substrates is limited to 2 min in each operation.

Materials and Methods

Protected dinucleotides with *o*-chlorophenylphosphodiesters were prepared according to Broka and coworkers.[24] MSNT, 1,1,3,3-tetramethylguanidine (TMG), and *syn*-pyridine-2-aldoximate (PAO) were purchased from Dojindo Laboratories. Reversed-phase chromatography was performed on alkylated silica gel (C_{18}, 55—105 μ, Waters Associates Inc.). HPLC was performed using a reversed-phase column (C_{18}, 5 μ, Senshu Pak). Ultraviolet (UV) spectra were measured on Hitachi model 200-10 spectrophotometer.

The Synthesis of Heptanucleotide——d-GATTCTA was synthesized using 37 mg of nucleoside resin (82 μmol/g), 15 mg of protected dimer (10—20 μmol) and MSNT (15 mg, 50 μmol) in pyridine (0.5 ml) according to Table I or Table I except for the detritylation step (20 min). The reactions were carried out in a small tube with a sintered glass filter.[11] After three coupling reactions, the resin was treated with 0.5 M TMG-PAO in dioxane–pyridine–water (5 : 4 : 1 v/v, 1.5 ml) for 36 h at room temperature. After evaporation of the volatile materials, the mixture was treated with a mixture of concentrated ammonia (2 ml) and pyridine (0.5 ml) at 55 °C for 4 h. The mixture was centrifuged and the resin was washed twice with aqueous pyridine (50% v/v). The combined solution was washed with ethyl acetate (6 ml) three times and evaporated. The residue was dissolved in 0.1 M triethylammonium acetate (TEAA, pH 7, 0.2 ml) and applied to a reversed phase (C_{18}) silica gel column (0.7 × 9 cm). Elution was carried out with a linear gradient made from 100 ml of 10% acetonitrile in 0.1 M TEAA (mixing vessel) and 100 ml of 30% acetonitrile in 0.1 M TEAA (reservoir) at a flow rate of 0.6 ml/min. The fractions containing the desired products were evaporated to a small volume, and 50% aq. acetic acid (5 ml) was added to the residue. After 15 min at room temperature, the mixture was washed with ether (5 ml) three times and evaporated. The heptamer with 72 A_{259} units from short acid treatment (total 6 min) or 63 A_{259} units from long acid treatment (total 42 min) was obtained. An aliquot of each heptamer was analyzed and further purified by reversed phase (C_{18}) silica gel HPLC. Elution was performed with a linear gradient made from 10% acetonitrile in 0.1 M TEAA and 20% acetonitrile in 0.1 M TEAA for 1 h at a flow rate of 1 ml per min (Fig. 2). Purified heptamer (5 A_{259} units) from Fig. 2b was hydrolyzed with snake venom phosphodiesterase. The mixture comprised 1 M triethylammonium bicarbonate (pH 7.5, 20 μl), H_2O (70 μl) and venom phosphodiesterase (mg/ml, 10 μl). After incubation at 37 °C for 4 h, the mixture was analyzed by paper electrophoresis (pH 3.5) to separate pC (0.82 A_{279} at pH 2), pA (1.76 A_{258} at pH 2), pT (1.70 A_{267} at pH 2) and G (0.82 A_{255} at pH 2). The ratio found was 1.0 : 2.0 : 2.9 : 1.1 (theoretical, 1 : 2 : 3 : 1).

The Synthesis of Pentadeca- and Nonadecanucleotides——d-A_{15}, d-AATGAGAAGCAACGA and d-AGAATCTCGTTGCTTCTCA were synthesized by the same procedure as described for the synthesis of the

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heptamer. After deprotection with 0.5 M TMG-PAO (1.5 ml) and concentrated ammonia (2.5 ml), the mixture was separated in the same way as used for the heptamer using the reversed phase (C_{18}) silica gel column (Fig. 2) and reversed phase HPLC (Fig. 4) after aqueous acetic acid treatment. In this way, 78 A_{257} units for d-A_{15}, 86 A_{256} units for d-AATGAGAAGCAACGA and 105 A_{259} units for d-AGAATCTCGTTGCTTCTCA were obtained. The purified pentadecamers (0.04 A_{260} units) were treated with [γ-^{32}P]ATP (4000 cpm/pmol, 100 μM, 1 μl) and T_4-polynucleotide kinase (1 unit/μl, 0.5 μl) in 5 μl of 50 mM Tris–HCl (pH 9.6), 1 mM $MgCl_2$, 2 mM spermine, 10 mM dithiothreitol and 0.1 M KCl at 37 °C for 30 min. An aliquot (about one tenth) was subjected to mobility shift analysis[22,23] (Fig. 5).

DNA Ligase Reaction——Purified d-GATTCTA (0.02 A_{260} units) was labeled using [γ-^{32}P]ATP and T_4-polynucleotide kinase as mentioned above. Without removing the excess [γ-^{32}P]ATP, labeled heptamer (0.01 A_{260} units, 150 pmol) and d-AATGAGAAGCAACGA (0.02 A_{260} units, 150 pmol) were mixed with d-AGAATCTCGTTGCTTCTCA (0.02 A_{260} units, 150 pmol) as a template in 18 μl of 66 mM Tris–HCl pH 7.6, 6.6 mM $MgCl_2$, and 500 μM ATP. The mixture was heated to 90 °C for 3 min and then cooled slowly to 15 °C over the period of 1 h. Then 1 μl of 0.2 M β-mercaptoethanol and 1 μl of T_4-DNA ligase (Takara Shuzo) (1.2 units/μl) were added to the mixture. The whole was incubated at 14—15 °C for 2 h, then an aliquot (3 μl) of the mixture was dissolved in 8 M urea, 10 mM ethylenediaminetetraacetic acid (EDTA), 40 mM Tris–borate (pH 8.4) containing 0.04% (w/v) marker dyes (3 μl) and loaded onto a 20% polyacrylamide gel electrophoresis at 400 V. The result is shown in Fig. 6.

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