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P-Loop Catalytically Assisting the Enzymatic Cleavage of Single-Stranded DNA

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Abstract—We demonstrated that a P-loop, a looped complex formed inside duplex DNA by adding peptide nucleic acids (PNA), acts catalytically as a template for enzymatic cleavage of single-stranded probe oligodeoxynucleotides (ODN). A PD-loop complex formed from P-loop and probe ODN was digested efficiently by a restriction enzyme, and the truncated probe ODN was released. The P-loop nicked by the enzyme can form PD-loop again with another probe ODN, and then assisted the enzymatic cleavage of an excess of probe ODN. In addition, by using dumbbell-formed ODN as a probe ODN, the efficiency of the P-loop-assisted ODN cleavage was enhanced considerably as compared with that of linear ODN. Thus, the method utilizing P-loop will make it possible to amplify the sequence information of duplex DNA via a catalytic cleavage of probe ODNs.
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Introduction

Peptide nucleic acids (PNAs) bind tightly to duplex DNA to produce an extended strand-displacement complex, P-loop. The displaced single-stranded DNA in P-loop is able to hybridize with a single-stranded DNA to form a PNA–DNA complex, PD-loop.[1–4] By using this unique property of PNAs, affinity capture[2,5] and topological labeling[6,7] of duplex DNA and highly selective detection of specific sequences[8,9] within duplex DNA have become possible. Recently, we reported a new method for the discrimination between cytosine and 5-methylcytosine in duplex DNA by sequence-selective formation of a fluorophore-labeled PD-loop, followed by the cleavage of the resulting complex with restriction enzymes.[10] The P- and PD-loops can be used as a facile and effective method for the selective detection of various sequences as well as for sequence-selective DNA modifications.

Herein, we report that P-loop catalytically acts as a template for enzymatic cleavage of single-stranded probe oligodeoxynucleotides (ODN). We observed that excess amount of probe ODN was cleaved by the combination of a PD-loop and a restriction enzyme. In addition, it was demonstrated that a dramatic enhancement of the cleavage efficiency was observed when the probe ODN had a dumbbell structure. Thus, the method utilizing P-loop makes it possible to amplify the sequence information of duplex DNA via catalytic cleavage of probe DNA.

Results and Discussion

DNAs, PNAs and probe ODNs used for enzymatic digestion of PD-loops are summarized in Table 1. PNAs were synthesized by standard solid-phase ᵗBOC peptide chemistry,[11] and PD-loops were prepared according to methods described elsewhere.[2] To avoid the binding of excess PNA with probe ODNs, PNA that did not hybridize with duplex DNA was removed from sample solutions by passage through a centrifugal filter. After filtration, the P-loop structure was confirmed by the oxidative degradation of thymine bases in P-loop by $KMnO_4$ treatment.[12] After hybridization with ^{32}P-5′-end-labeled probe ODN, the PD-loop was incubated with the restriction enzyme *Hha* I (Fig. 1).

Initially, we examined the enzymatic digestion of PD-loop containing 10-mer **PNA 1**, 18-mer **ODN 1** and 80-mer DNA duplex (**DNA 1**/**DNA 1′**) which has a recognition site of *Hha* I (5′-GCGC-3′). A 10-fold excess amount of ^{32}P-5′-end-labeled **ODN 1** was added to a

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Table 1. DNA and PNA oligomers used in this study

		Sequences
Duplex DNAs[a,b]		
	DNA 1	5′-(24 mer[c])*TTTTTTTTTT*GTCA**GCGC**ATGG*TTTTTTTTTT*(24 mer[d])-3′
	DNA 1′	Complementary to **DNA 1**
	DNA 1(5′)	5′-(24 mer[c])*TTTTTTTTTT*GTCA**GCG**-3′
	DNA 1(3′)	5′-p**C**ATGG*TTTTTTTTTT*(24 mer[d])-3′
	DNA 1(mC)	5′-(24 mer[c])*TTTTTTTTTT*GTCA**G^{m}CGC**ATGG*TTTTTTTTTT*(24 mer[d])-3′
	DNA 2	5′-(24 mer[e])*GTGTCCAG*CTCA**GCGC**ATGG*GTGTCCAG*(24 mer[f])-3′
	DNA 2′	Complementary to **DNA 4**
Probe ODNs[a,g]		
	ODN 1	5′-AAACCAT**GCGC**TGACAAA-3′
	ODN 2	5′-CACCCAT**GCGC**TGAGCTG-3′
	ODN 3	5′-CATGGAAAACCAT**GCGC**TGACAAAAGTCAG-3′
PNA oligomers[h]		
	PNA 1	H-TTTTTTTTTT-NH_2
	PNA 2	H-GTGTCCAG-NH_2

[a]Recognition sites of restriction enzyme *Hha* I are shown in bold.
[b]PNA binding sites are shown in italics.
[c]5′-TCATCCTCGGCACCGTCACCCTGG-3′.
[d]5′-AGCCACTATCGACTATCATGGCGA-3′.
[e]5′-TCATGCTCGGCACCGTCACCCTGC-3′.
[f]5′-AGCGACTATCGACTATCATGGCGA-3′.
[g]Stem sites of dumbbell structure are underlined.
[h]PNA is written from N- to C-terminal using normal peptide conventions: 'H' denotes a free amino group, while 'NH_2' denotes a terminal carboxyamide.

solution of P-loop consisting of **DNA 1**, **DNA 1′** and **PNA 1**, and then the digestion of the resulting PD-loop by restriction enzyme *Hha* I was carried out. Figure 2 shows the representative gel electrophoresis showing cleavage efficiency. In the absence of **PNA 1**, no cleavage of **ODN 1** was observed (lane 1), indicating that **ODN 1** was not recognized as a substrate for *Hha* I. In contrast, the cleavage efficiency of **ODN 1** in the presence of **PNA 1** remarkably increased with increasing incubation time (lanes 2–4). This result indicates that the PD-loop, which was formed via strand invasion by **PNA 1**, can be a substrate for *Hha* I. The k_{obs} of **ODN 1** cleavage was calculated to be 0.17 nM/s. Lane 4 of Figure 2 shows that 67% of **ODN 1** was cleaved through enzymatic digestion for 1 h (lane 4). As calculated from the concentration of ODN and P-loop in the reaction mixture, 67% cleavage of **ODN 1** implies that 6.7-fold excess of **ODN 1** relative to the P-loop complex is consumed by the restriction enzyme. Thus, the 'nicked' P-loop, which has been used for enzymatic digestion of **ODN 1**, is likely to bind again to another **ODN 1**, and can be repeatedly used as a template for the digestion of excess **ODN 1**.

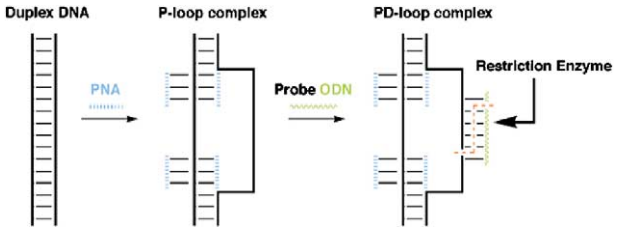

Figure 1. Schematic illustration of the formation and enzymatic digestion of a PD-loop that has two PNA-binding sites and a probe ODN-binding site.

As a control experiment, we examined the enzymatic cleavage of **ODN 1**/**DNA 1** duplex. Also in this experiment, an excess amount of **ODN 1** relative to **DNA 1** was cleaved by *Hha* I (1.4 equiv of **ODN 1** to P-loop in 30 min, and 4.7 equiv in 1 h). However, the cleavage efficiency of **ODN 1**/**DNA 1** duplex was considerably lower than that observed for the PD-loop as shown in

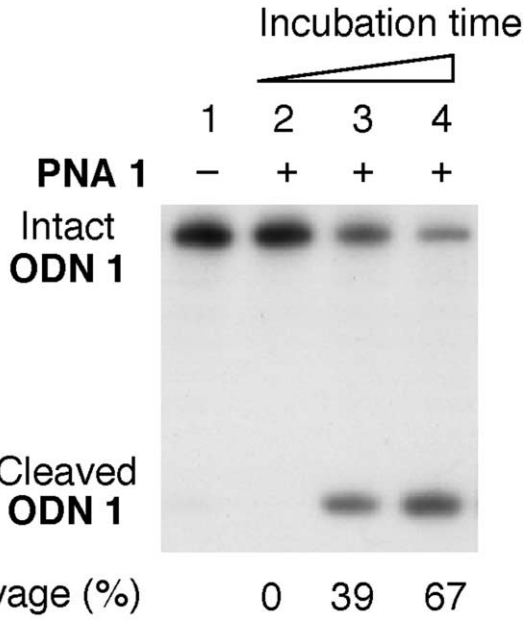

Figure 2. An autoradiogram of a denaturing 15% polyacrylamide electrophoresis gel of an enzymatic digest of the PD-loop consisting of **DNA 1**, **DNA 1′**, **PNA 1** and ^{32}P-5′-end-labeled **ODN 1**. The P-loop (90 nM) incubated with 900 nM of **ODN 1** was treated with *Hha* I (10 units) at 37 °C for 0 (lane 2), 30 (lane 3) or 60 min (lanes 1 and 4). The presence of **PNA 1** is indicated by (+) (lanes 2–4), and the absence is indicated by (−) (lane 1).

Figure 2. These results indicate that the nicked P-loop acts as an effective template for catalytic cleavage of **ODN 1**. Since **DNA 1′** would keep both ends of nicked **DNA 1** fragments in close proximity to each other, **ODN 1** would bind to nicked **DNA 1** efficiently, resulting in an efficient cleavage of **ODN 1**.

The proposed reaction mechanism for ODN cleavage, based on the experimental results described above, is shown in Figure 3. The nicked P-loop hybridizes with probe ODN repeatedly as a catalytic template. We prepared the nicked P-loop using **DNA 1(5′)**, **DNA 1(3′)**, **DNA 1′** and **PNA 1**, and investigated the enzymatic cleavage of **ODN 1** (Fig. 4). The nicked P-loop also efficiently assisted the cleavage of an excess amount of **ODN 1** (4.9-fold over the nicked P-loop in 1 h) as observed for the P-loop-assisted cleavage, suggesting

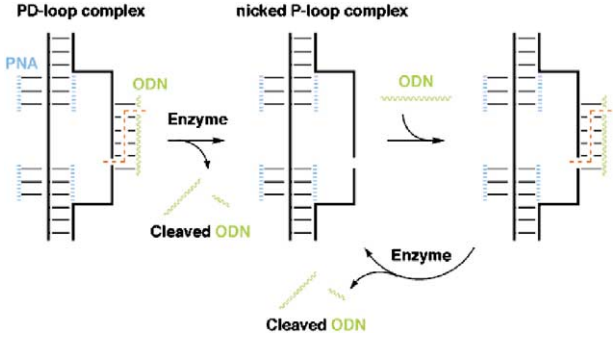

Figure 3. Proposed mechanism for the enzymatic digestion of ODN by a nicked P-loop. The nicked P-loop acts as a catalytic template during the digestion.

that the nicked complex can act again as an effective template for the enzymatic cleavage of **ODN 1**, as shown in Figure 3.

In order to elucidate the importance of P-loop template for enzymatic ODN cleavage, we investigated the cleavage efficiency of the probe ODN using the P-loop containing a G^{m}CGC sequence at the site recognized by the restriction enzyme *Hha* I (**DNA 1(**^{m}**C)**/**DNA 1′**/**PNA 1**, ^{m}C denotes 5-methylcytosine). Only 6% of the ODN was cleaved in 1 h incubation, indicating that enzymatic ODN cleavage was strongly suppressed by P-loop methylation (Fig. 5). This result strongly suggests that *Hha* I recognizes the PD-loop formed by the P-loop and the probe ODN to give the cleavage products. P-loop acts as an effective template that assists the enzymatic ODN cleavage.

The possibility of ODN cleavage via a PD-loop, that did not contain a homopurine/homopyrimidine sequence for a PNA-binding site, was also investigated. In this experiment, the P-loop containing GTGTCCAG sequence replaced by 8-mer PNA (**DNA 2**/**DNA 2′**/**PNA 2**) was incubated with **ODN 2** (Fig. 6). Although the cleavage efficiency for **ODN 2** was not so high as compared with the case for **DNA 1**/**DNA 1′** containing homopurine/homopyrimidine sequences, the cleavage of **ODN 2** was observed clearly by PAGE analysis. The efficiency of enzymatic cleavage assisted by P-loop seems to be strongly influenced by the sequences restrictions imposed by the target sequences and the well described limitations for duplex invasion by PNAs.[4]

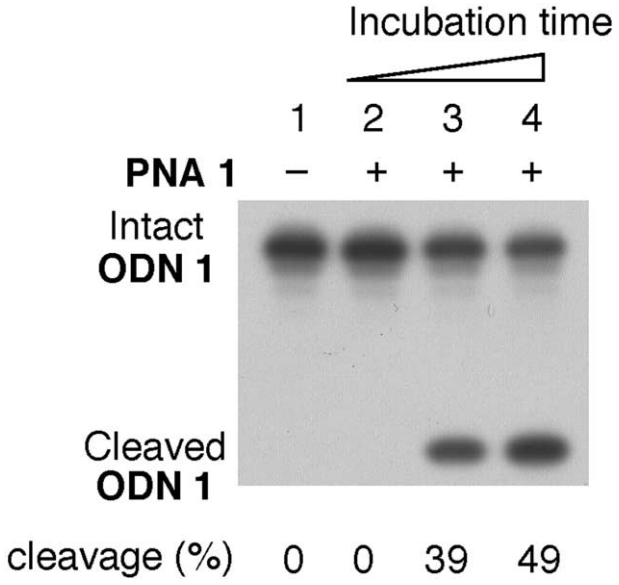

Figure 4. Enzymatic digestion of **ODN 1** using a nicked P-loop template. An autoradiogram of a denaturing 15% polyacrylamide electrophoresis gel of an enzymatic digest of the PD-loop consisting of **DNA 1(5′)**, **DNA 1(3′)**, **DNA 1′**, **PNA 1** and ^{32}P-5′-end-labeled **ODN 1**. The nicked P-loop (90 nM) incubated with 900 nM of **ODN 1** was treated with *Hha* I (10 units) at 37 °C for 0 (lanes 2), 30 (lanes 3), or 60 min (lanes 1 and 4). The presence of **PNA 1** is indicated by (+) (lanes 2–4), and the absence is indicated by (−) (lane 1).

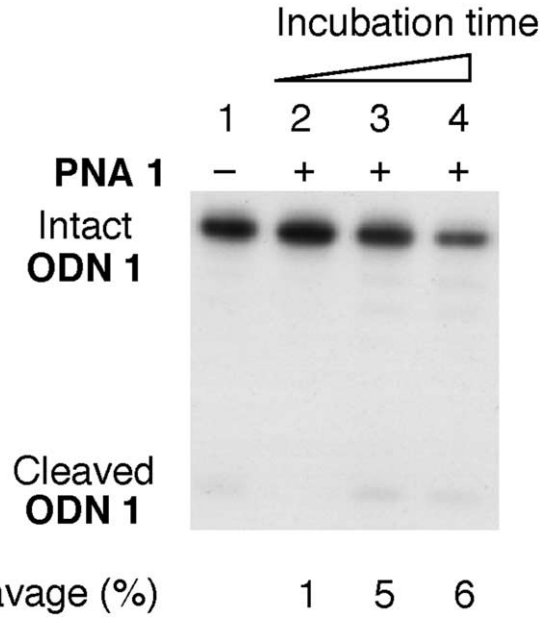

Figure 5. Inefficient cleavage of probe ODN using methylated P-loop as a template for enzymatic digestion. Shown is an autoradiogram of a denaturing 15% polyacrylamide electrophoresis gel of an enzymatic digest of the PD-loop consisting of **DNA 1(**^{m}**C)**, **DNA 1′**, **PNA 1** and ^{32}P-5′-end-labeled **ODN 1**. The P-loop (90 nM) incubated with 900 nM of **ODN 1** was treated with *Hha* I (10 units) at 37 °C for 0 (lane 2), 30 (lane 3) or 60 min (lanes 1 and 4). The presence of **PNA 1** is indicated by (+) (lanes 2–4), and the absence is indicated by (−) (lane 1).

In order to improve the efficiency of enzymatic digestion of the PD-loop, we next examined the enzymatic digestion of dumbbell-formed **ODN 3** hybridized with the P-loop consisting of **DNA 1**, **DNA 1′** and **PNA 1**. Because the enzymatic digestion of **ODN 3** should give two hairpin ODNs, it was expected that the inhibition of catalytic cleavage of ODN due to the rebinding of the cleaved ODN to the template P-loop would be suppressed (Fig. 7A). One, 10, 50, or 100-equivalents of **ODN 3** were added to a solution of the P-loop, and subsequently *Hha* I was added to the mixture. A representative gel is shown in Figure 7B. Analysis of the enzymatic digest using **ODN 3** revealed that 47-fold excess amount of **ODN 3** relative to P-loop complex was cleaved in 1 h-incubation with *Hha* I (lane 4). The k_{obs} of ODN cleavage was calculated to be 1.18 nM/s. The P-loop-assisted enzymatic digestion of **ODN 3** was greatly accelerated, as compared with the case for the enzymatic digestion of linear **ODN 1** (6.7-fold excess amount of **ODN 1** to P-loop complex).

Conclusion

In the present study, we demonstrated that P-loop can act as an excellent template for the digestion of probe ODNs by restriction enzymes. P-loops nicked by the enzyme also catalytically assist the cleavage of the probe ODN. In addition, by using dumbbell-formed probe ODN, cleavage of dumbbell ODN proceeded more efficiently than that for linear ODN. Thus, the P-loop, assisting the continuous enzymatic cleavage of the strand that is complementary for the sequence in the duplex, can amplify the information gained about specific gene sequences via the cleavage of a large amount of complementary probe ODN. P-loops can also be used as powerful tools for gene analysis, typing of cytosine methylation, and sequence-specific gene modification.

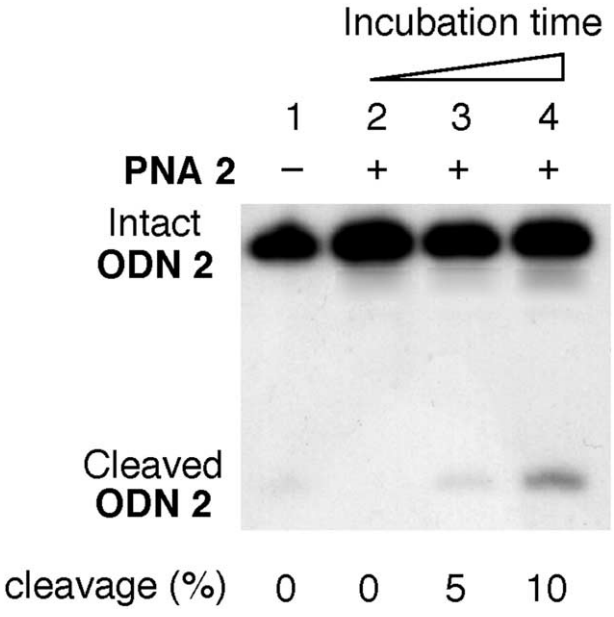

Figure 6. An autoradiogram of a denaturing 15% polyacrylamide electrophoresis gel of an enzymatic digest of the PD-loop consisting of **DNA 2**, **DNA 2′**, **PNA 2** and ^{32}P-5′-end-labeled **ODN 2**. The P-loop (90 nM) incubated with 900 nM of **ODN 2** was treated with *Hha* I (10 units) at 37 °C for 0 (lane 2), 30 (lane 3) or 60 min (lanes 1 and 4). The presence of **PNA 2** is indicated by (+) (lanes 2–4), and the absence is indicated by (−) (lane 1).

Experimental

PNA

PNA was synthesized with a conventional ′BOC strategy on a solid support synthesis using MBHA resin as described.[11,13] H-TTTTTTTTTT-NH_2 (**PNA 1**): MALDI-TOF [$(M+H)^+$] calcd 2680.60, found 2681.60. H-GTGTCCAG-NH_2 (**PNA 2**): MALDI-TOF [$(M+H)^+$] calcd 2202.12, found 2202.82.

Preparation of ^{32}P-5′-end-labeled DNA

DNA was 5′-end-labeled by phosphorylation with 4 μL [γ-^{32}P]ATP and 4 μL T4 polynucleotide kinase using standard procedures.[14] The 5′-end-labeled DNA was recovered by ethanol precipitation and further purified by 15% nondenaturing gel electrophoresis, then isolated by the crush and soak method.[15]

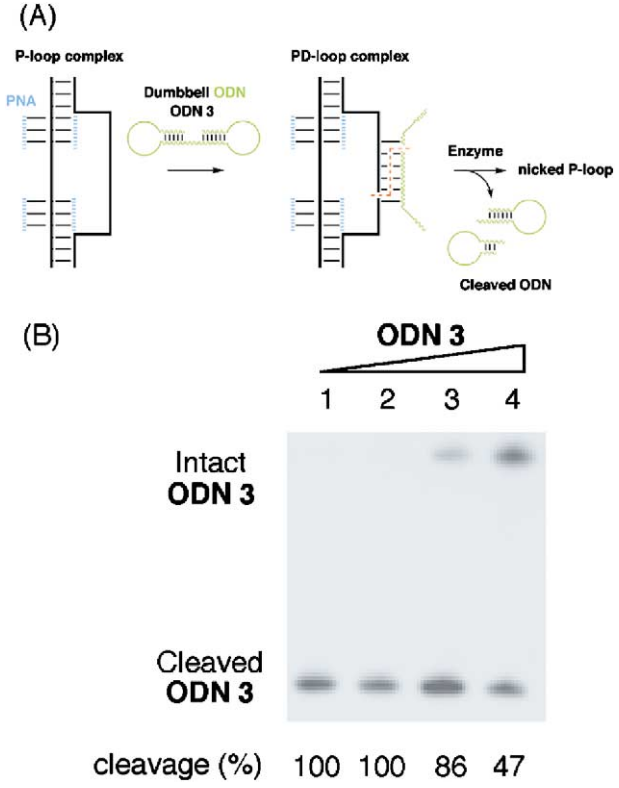

Figure 7. Cleavage of dumbbell-formed ODN: (A) schematic illustration of the enzymatic digestion of the PD-loop containing ^{32}P-5′-end-labeled dumbbell-formed **ODN 3**; (B) an autoradiogram of a denaturing 15% polyacrylamide electrophoresis gel of the enzymatic digest of the PD-loop consisting of **DNA 1**, **DNA 1′**, **PNA 1** and ^{32}P-5′-end-labeled **ODN 3**. The P-loop (90 nM) incubated with 90 (lane 1), 900 (lane 2), 4500 (lane 3) or 9000 nM of **ODN 3** was treated with *Hha* I (10 units) at 37 °C for 1 h.

General protocol for the cleavage of ^{32}P-labeled ODN 1 by *Hha* I using P-loop

Enzymatic digestion experiments were executed in a total volume of 20.0 or 22.2 μL with final concentrations of each species as indicated. Binding of PNA to 90 or 100 nM duplex DNA was carried out at 37 °C for 4 h with the corresponding PNA (200 equiv to DNA duplex). Excess PNA was removed from samples by centrifugation with Microcon® (YM-30, 30,000 NMWL), centrifugal filter devices, at 0 °C. After centrifugation, the mixture was incubated with ^{32}P-5′-end-labeled **ODN 1** at 37 °C for 1 h. The resulting PD-loop was incubated in 10 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT and 50 mM NaCl with *Hha* I (10 U) at 37 °C. The reaction was quenched by the addition of 3 μL of a solution containing SDS (1%), glycerol (50%) and bromophenol blue (0.05%). The reaction mixture was ethanol precipitated with 800 μL ethanol. The precipitated DNA was washed with 100 μL cold ethanol and dried in vacuo. The radioactivity of the samples was measured using an Aloka 1000 liquid scintillation counter, and the dried DNA pellets were resuspended in 80% formamide loading buffer (a solution of 80% v/v formamide, 1 mM EDTA, 0.1% xylene cyanol and 0.1% bromophenol blue). The samples (1 mL, 10×10^3 cpm) were loaded onto 15% polyacrylamide and 7 M urea sequencing gel, and electrophoresed at 1900 V for approximately 1 h. The gel was dried and exposed to X-ray film with an intensifying sheet at −80 °C. Cleavage of the labeled strand was quantified by phosphorimagery using Molecular Analyst software (version 2.1).

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