Title
Replication of Pyridyloxobutyl Phosphotriester Lesions in Cells.

Permalink
https://escholarship.org/uc/item/9db492zv

Journal
Chemical Research in Toxicology, 33(2)

Authors
Wu, Jiabin
Wang, Yinsheng

Publication Date
2020-02-17

DOI
10.1021/acs.chemrestox.9b00485

Peer reviewed

Replication of Pyridyloxobutyl Phosphotriester Lesions in Cells

Jiabin Wu,

University of California, Riverside, California

Yinsheng Wang

University of California, Riverside, California

Abstract

Genome integrity is constantly challenged by endogenous or exogenous genotoxic agents, which can give rise to various DNA adducts. After metabolic activation, tobacco-specific nitrosamines N'-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) can lead to pyridyloxobutylphosphotriesters (POB-PTEs) in DNA. Here, we synthesized oligodeoxyribonucleotides containing a site-specifically inserted S_P- or R_P-POB-PTE flanked by two thymidines, and we examined the impact that these lesions have on DNA replication in *Escherichia coli* cells. We found that these two lesions are not strong impediments to DNA replication, and their replicative bypass is not modulated by genetic depletion of the three SOS-induced DNA polymerases or Ada protein. In addition, neither S_P- nor R_P-POB-PTEs was mutagenic in *E. coli* cells. Together, our study unveiled, for the first time, the influence of tobacco-specific nitrosamine-induced POB-PTE lesions on DNA replication *in vivo*.

Graphical Abstract

Translesion synthesis
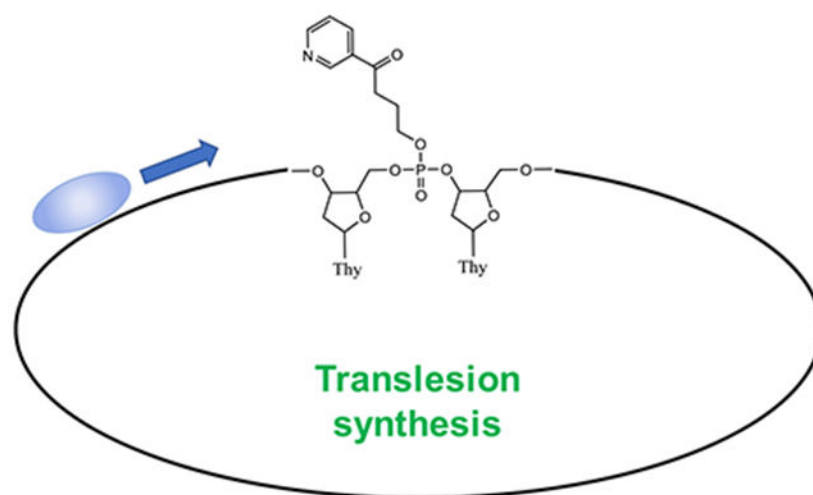
Owing to its chemical instability, DNA is susceptible to damage by endogenous and exogenous factors, resulting in the formation of various DNA adducts.[1,2] If not efficiently repaired, these DNA adducts may induce mutagenesis and ultimately carcinogenesis.[3] Tobacco and its combustion products contain more than 8000 compounds,[4] and over 70 of them are classified as carcinogens by the International Agency for Research on Cancer (IARC).[5] Among them, two tobacco-specific nitrosamines, N'-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), are rated as group-I carcinogens (i.e., carcinogenic to humans) by the IARC.[6] Additionally, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), a reduced metabolite of NNK, was found to induce cancer in rodents.[7–9]

The carcinogenic effects of NNN and NNK are thought to arise from their capabilities in inducing DNA adducts and eliciting mutagenesis. In particular, NNN and NNK can be metabolically activated by cytochrome P450s to yield reactive intermediates that can pyridyloxobutylate DNA.[9–11] Among the DNA lesions induced by NNN and NNK, O^2-pyridyloxobutyl-thymidine (O^2-POB-dT) and O^6-POB-2′-deoxyguanosine (O^6-POB-dG) could accumulate at substantial levels in lung tissues of NNK-treated mice, and O^2-POB-cytosine and $N7$-POB-guanine were also detected as nucleobase adducts.[12] Additionally, POB-nucleobase adducts were found to be mutagenic *in vivo*. O^2-POB-dT elicits T → A, T → C, and T → G mutations in *E. coli* cells.[13] O^6-POBdG induces G → A mutation in *E. coli* cells and both G → A and G → T mutations in HEK293T cells.[14,15] Du et al.[16] also revealed that, in HEK293T cells, O^2-POBdT and O^4-POBdT direct T → A and T → C mutations, respectively.

Apart from nucleobase adducts, backbone alkylation products, also known as alkyl phosphotriesters (alkyl-PTEs), can be induced at appreciable levels and are persistent in mammalian tissues.[17] Many methylating agents, for example, *N*-methyl-*N*-nitrosourea, diazomethane, and methylmethanesulfonate, could attack noncarbon-bonded oxygen atoms at the backbone phosphate to yield methyl-PTEs.[18–20] Likewise, reactive metabolites of NNN and NNK can attack backbone phosphate groups in DNA to form pyridyloxobutyl phosphotriesters (POB-PTEs) *in vitro* and in rats (Figure 1).[21,22] In addition, PTEs

constitute the major alkylation products of DNA induced by *N*-ethyl-*N*-nitrosourea,[23] and 55–73% of the total POB DNA adducts induced by NNK reside on the phosphate backbone, [21] suggesting that POB-PTEs can serve as biomarkers for some tobacco-related diseases, for example, lung cancer.[21] Furthermore, POB-PTEs appear to be poorly repaired in mammalian tissues, and these lesions remain highly abundant for over 70 weeks in lung tissue of rats treated with drinking water containing 5 ppm of NNN.[22]

Since the alkyl group could neutralize the negative charge on the phosphate backbone, PTE lesions were shown to perturb the interactions of DNA with DNA-binding proteins including MutS,[24] MutY,[25] and a RNA polymerase.[26] Results from *in vitro* primer extension assay showed that Et-PTE-containing oligodeoxyribonucleotide (ODN) inhibits *in vitro* replication mediated by purified T4 DNA polymerase and *E. coli* DNA polymerase I.[27] Our previous replication study in *Escherichia coli* cells also revealed that replication across S_P-Me-PTE at flanking TT dinucleoside site elicits TT → GT and TT → GC mutations, which necessitate the Ada protein.[28] However, no research has been conducted to examine how POB-PTEs affect DNA replication *in vitro* or in cells.

The objectives of this study were to assess the impact of POB-PTEs on DNA replication in bacterial cells, to define the roles of the three SOS-induced DNA polymerases in bypassing these lesions, and to examine the function of Ada in modulating the replication across the POB-PTEs in *E. coli* cells.

We synthesized 12-mer ODNs containing a site-specifically inserted and stereochemically defined POB-PTE lesion. The synthesized ODNs were purified by HPLC and characterized with ESI-MS and MS/MS (Scheme 1, Figures S1 and S2). Our previous results revealed that, by using a C18 reversed-phase column with triethylammonium acetate/acetonitrile as mobile phases, the S_P-Me-PTE-containing ODN elutes earlier than the corresponding R_P-Me-PTE-harboring ODN.[28] Here, we also assigned the earlier- and later-eluting fractions as S_P- and R_P-POB-PTE-harboring ODNs, respectively (Figure S1). The MS/MS results confirmed the sequence of the synthesized ODNs and verified the location of phosphate group with which the POB was conjugated (Figure S2).

We next employed a competitive replication and adduct bypass (CRAB) assay to assess the bypass efficiencies of the POB-PTE lesions.[29] A mixture with a fixed ratio of lesion-containing and competitor vectors was transfected into AB1157 cells. After replication, the progeny plasmids were extracted and PCR amplified, and the resultant PCR amplicons were digested with restriction endonucleases (Figure S3). In this vein, *Bbs*I and *Mlu*CI were employed to digest the PCR products to yield 10mer ODNs, which contain the replication products of the initial damage-containing site for lesion-containing or lesion-free control genome, or 13mer for the corresponding competitor genome, of which the 5′-termini were radiolabeled (Figure S3). By switching the order of digestion for the two restriction enzymes, the lesion-situated strand (p*GGCMNGCTAT) or the corresponding complementary strand (p*AATTATAGCY) can be selectively radiolabeled, where 'p*' denotes the radiolabeled phosphate, 'M' and 'N' designate the nucleobases at the two nucleotides initially flanking the PTE base, and 'Y' indicates the nucleobase opposite 'N'. The resulting ODNs were analyzed by native PAGE and LC–MS/MS (Figures S3–S5).

Alkylation of the phosphate backbone can potentially influence the fidelity of replication at both nucleosides flanking the PTE site,[28] yielding up to 16 replication products (with A, T, C, or G being incorporated at the two flanking nucleoside sites, Figure S4), which cannot be fully separated from each other by PAGE. Therefore, we subjected the aforementioned digestion products to LC–MS and MS/MS analyses, where we monitored the $[M-3H]^{3-}$ ions of GGCMNGCTAT. The LC–MS/MS data showed that neither S_P- nor R_P-POB-PTE was mutagenic, which is consistent with the PAGE results (Figures S4 and S5).

After the replication products were identified, we determined the bypass efficiencies of POB-PTEs by comparing the relative intensity ratio of 10-mer band from the lesion-containing genome over the corresponding 13-mer band from the competitor genome (Figure 1). The results showed that neither S_P- nor R_P-POB-PTE inhibits DNA replication. The slightly higher replication bypass efficiency for the S_P diastereomer than the R_P counterpart could be attributed to the difference in structural alterations to DNA induced by the two diastereomers and the ensuing differential recognition of the two diastereomers by DNA polymerase(s). In this regard, the S_P diastereomer is known to point perpendicularly out from the DNA double helix, whereas the R_P diastereomer projects into the major groove. [17]

We also assessed the potential roles of the three SOS-induced DNA polymerases (Pol II, Pol IV, and Pol V) in bypassing the POB-PTEs by conducting the CRAB assay using isogenic *E. coli* strains deficient in Pol II, Pol IV, Pol V, or all three in combination. The result showed that the replicative bypass of POB-PTEs did not require any of the SOS-induced DNA polymerases (Figure 1).

Previous research has shown that *E. coli* Ada protein could remove S_P-Me-PTE and is required for the mutagenic bypass of the S_P-Me-PTE at TT site.[28,30] Thus, we next asked if Ada protein can influence the bypass efficiency and mutation patterns of the bulky POB-PTEs, and we observed that genetic ablation of Ada protein did not elicit any alterations in the replication efficiency. Moreover, replication across neither diastereomers of POB-PTEs is mutagenic in the Ada-deficient background (Figure 1).

It is worth discussing the findings made from the present study. First, we revealed that the S_P- and R_P-POB-PTEs flanked by two thymidines are well tolerated by DNA polymerases in cells, where no appreciable perturbation in DNA replication efficiency or accuracy is observed; hence, the POB-PTEs at this flanking sequence are rather benign lesions in terms of their impact on DNA replication in *E. coli* cells. It will be important to examine, in the future, whether the same findings can be extended to these lesions in other flanking nucleoside sequences.

Second, we observed that the bypass efficiencies of the POB-PTEs are not impacted by the genetic depletion of Ada, or SOS-induced DNA polymerases (Pol II, Pol IV, and Pol V, alone or all three together), and the replication across these lesions is not mutagenic in any of the genetic backgrounds we examined. This finding supports that replication across these lesions does not require these TLS polymerases, which parallels what we observed previously for simple alkyl-PTEs with various sizes of alkyl groups and different

stereochemical configurations of the PTEs.[28] Previous research revealed that *E. coli* Ada protein can remove S_P-Me-PTE at TT dinucleotide site, albeit at the expense of eliciting mutations.[28,30] The absence of influence of Ada on the efficiency of replication across the POB-PTEs suggests the lack of interaction between Ada and POB-PTEs.

In summary, our study provides, for the first time, the knowledge about how bulky POB group on phosphate backbone influences DNA replication in bacterial cells. Future studies about how POB-PTEs affect DNA replication and transcription in mammalian cells, and how they are repaired in mammalian cells, will offer additional insights into the biological impacts of this unique class of DNA damage. The POB-PTE-bearing ODNs synthesized herein set the stage for these studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

REFERENCES

(1). Fu D, Calvo JA, and Samson LD (2012) Balancing repair and tolerance of DNA damage caused by alkylating agents. Nat. Rev. Cancer 12, 104–120. [PubMed: 22237395]

(2). Lindahl T, and Wood RD (1999) Quality control by DNA repair. Science 286, 1897–1905. [PubMed: 10583946]

(3). Shrivastav N, Li D, and Essigmann JM (2010) Chemical biology of mutagenesis and DNA repair: cellular responses to DNA alkylation. Carcinogenesis 31, 59–70. [PubMed: 19875697]

(4). Rodgman A, and Perfetti TA (2016) The Chemical Components of Tobacco and Tobacco Smoke, CRC Press.

(5). Hecht SS (2012) Research opportunities related to establishing standards for tobacco products under the Family Smoking Prevention and Tobacco Control Act. Nicotine Tob. Res 14, 18–28. [PubMed: 21324834]

(6). Ma B, Zarth AT, Carlson ES, Villalta PW, Upadhyaya P, Stepanov I, and Hecht SS (2018) Methyl DNA phosphate adduct formation in rats treated chronically with 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and enantiomers of its metabolite 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol. Chem. Res. Toxicol 31, 48–57. [PubMed: 29131934]

(7). Hoffmann D, and Hecht SS (1985) Nicotine-derived *N*-nitrosamines and tobacco-related cancer-current status and future-directions. Cancer Res. 45, 935–944. [PubMed: 3882226]

(8). Hecht SS, and Hoffmann D (1988) Tobacco-specific nitrosamines, an important group of carcinogens in tobacco and tobacco-smoke. Carcinogenesis 9, 875–884. [PubMed: 3286030]

(9). Hecht SS (1998) Biochemistry, biology, and carcinogenicity of tobacco-specific *N*-nitrosamines. Chem. Res. Toxicol 11, 559–603. [PubMed: 9625726]

(10). Hecht SS (2003) Tobacco carcinogens, their biomarkers and tobacco-induced cancer. Nat. Rev. Cancer 3, 733. [PubMed: 14570033]

(11). Hecht SS, Stepanov I, and Carmella SG (2016) Exposure and metabolic activation biomarkers of carcinogenic tobacco-specific nitrosamines. Acc. Chem. Res 49, 106–114. [PubMed: 26678241]

(12). Hecht SS (1999) DNA adduct formation from tobacco-specific *N*-nitrosamines. Mutat. Res., Fundam. Mol. Mech. Mutagen 424, 127–142.

(13). Jasti VP, Spratt TE, and Basu AK (2011) Tobacco-specific nitrosamine-derived O^2-alkylthymidines are potent mutagenic lesions in SOS-induced *Escherichia coli*. Chem. Res. Toxicol 24, 1833–1835. [PubMed: 22029400]

(14). Pauly GT, Peterson LA, and Moschel RC (2002) Mutagenesis by O(6)-[4-oxo-4-(3-pyridyl)butyl]guanine in *Escherichia coli* and human cells. Chem. Res. Toxicol 15, 165–169. [PubMed: 11849042]

(15). Weerasooriya S, Jasti VP, Bose A, Spratt TE, and Basu AK (2015) Roles of translesion synthesis DNA polymerases in the potent mutagenicity of tobacco-specific nitrosamine-derived O^2-alkylthymidines in human cells. DNA Repair 35, 63–70. [PubMed: 26460881]

(16). Du H, Leng J, Wang P, Li L, and Wang Y (2018) Impact of tobacco-specific nitrosamine-derived DNA adducts on the efficiency and fidelity of DNA replication in human cells. J. Biol. Chem 293, 11100–11108. [PubMed: 29789427]

(17). Jones GD, Le Pla RC, and Farmer PB (2010) Phosphotriester adducts (PTEs): DNA's overlooked lesion. Mutagenesis 25, 3–16. [PubMed: 19920061]

(18). Brimacombe R, Griffin BE, Haines J, Haslam WJ, and Reese C (1965) An approach to the methylation of polynucleotides. Biochemistry 4, 2452–2458.

(19). Bannon P, and Verly W (1972) Alkylation of phosphates and stability of phosphate triesters in DNA. Eur. J. Biochem 31, 103–111. [PubMed: 4344908]

(20). Lawley PD (1973) Reaction of *N*-methyl-*N*-nitrosourea (MNUA) with ^{32}P-labelled DNA: evidence for formation of phosphotriesters. Chem.-Biol. Interact 7, 127–130. [PubMed: 4588104]

(21). Li Y, Ma B, Cao Q, Balbo S, Zhao L, Upadhyaya P, and Hecht SS (2019) Mass spectrometric quantitation of pyridyloxobutyl DNA phosphate adducts in rats chronically treated with *N*-nitrosonornicotine. Chem. Res. Toxicol 32, 773–783. [PubMed: 30740971]

(22). Ma B, Villalta PW, Zarth AT, Kotandeniya D, Upadhyaya P, Stepanov I, and Hecht SS (2015) Comprehensive high-resolution mass spectrometric analysis of DNA phosphate adducts formed by the tobacco-specific lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. Chem. Res. Toxicol 28, 2151–2159. [PubMed: 26398225]

(23). Beranek DT, Weis CC, and Swenson DH (1980) A comprehensive quantitative-analysis of methylated and ethylated DNA using high-pressure liquid-chromatography. Carcinogenesis 1, 595–606. [PubMed: 11219835]

(24). Biswas I, and Hsieh P (1997) Interaction of MutS protein with the major and minor grooves of a heteroduplex DNA. J. Biol. Chem 272, 13355–13364. [PubMed: 9148958]

(25). Lu AL, Tsai-Wu JJ, and Cillo J (1995) DNA determinants and substrate specificities of *Escherichia coli* MutY. J. Biol. Chem 270, 23582–23588. [PubMed: 7559523]

(26). Marushige K, and Marushige Y (1983) Template properties of DNA alkylated with *N*-methyl-*N*-nitrosourea and *N*-ethyl-*N*-nitrosourea. Chem.-Biol. Interact 46, 179–188. [PubMed: 6354490]

(27). Tsujikawa L, Weinfield M, and Reha-Krantz LJ (2003) Differences in replication of a DNA template containing an ethyl phosphotriester by T4 DNA polymerase and *Escherichia coli* DNA polymerase I. Nucleic Acids Res. 31, 4965–4972. [PubMed: 12930945]

(28). Wu J, Wang P, and Wang Y (2018) Cytotoxic and mutagenic properties of alkyl phosphotriester lesions in *Escherichia coli* cells. Nucleic Acids Res. 46, 4013–4021. [PubMed: 29514270]

(29). Delaney JC, and Essigmann JM (2006) Assays for determining lesion bypass efficiency and mutagenicity of site-specific DNA lesions *in vivo*. Methods Enzymol. 408, 1–15. [PubMed: 16793359]

(30). Weinfeld M, Drake AF, Saunders JK, and Paterson MC (1985) Stereospecific removal of methyl phosphotriesters from DNA by an *Escherichia coli ada*+ extract. Nucleic Acids Res. 13, 7067–7077. [PubMed: 3903661]
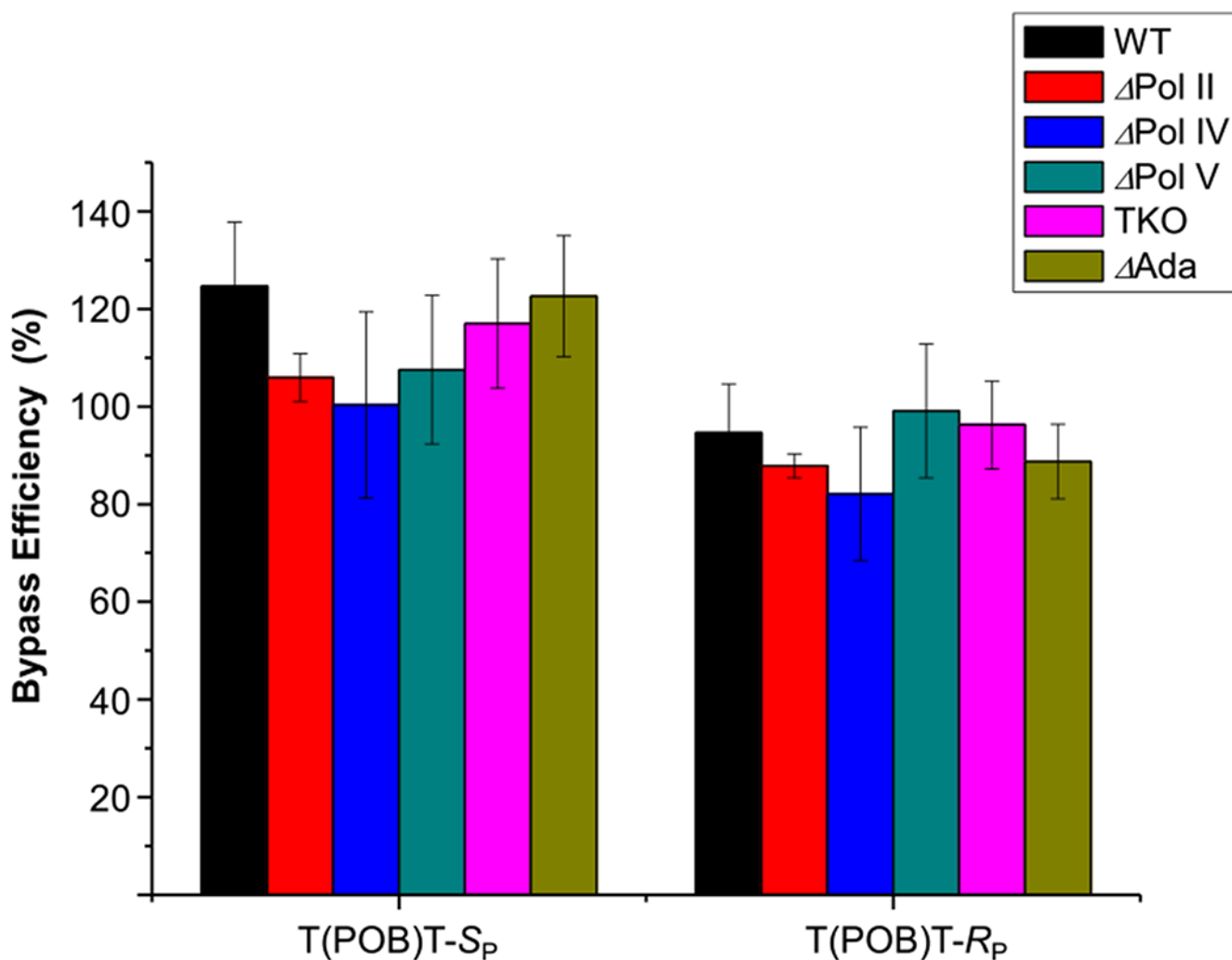
Figure 1.
Bypass efficiencies of POB-PTEs in AB1157 *E. coli* strains that are proficient in translesion synthesis and Ada, or with Ada protein being genetically ablated, or the three SOS-induced DNA polymerases (Pol II, Pol IV, and Pol V) being individually or simultaneously knocked out. "TKO" designates triple knockout of all three SOS-induced polymerases. The data represent the mean and standard deviation of results from three independent replication experiments.
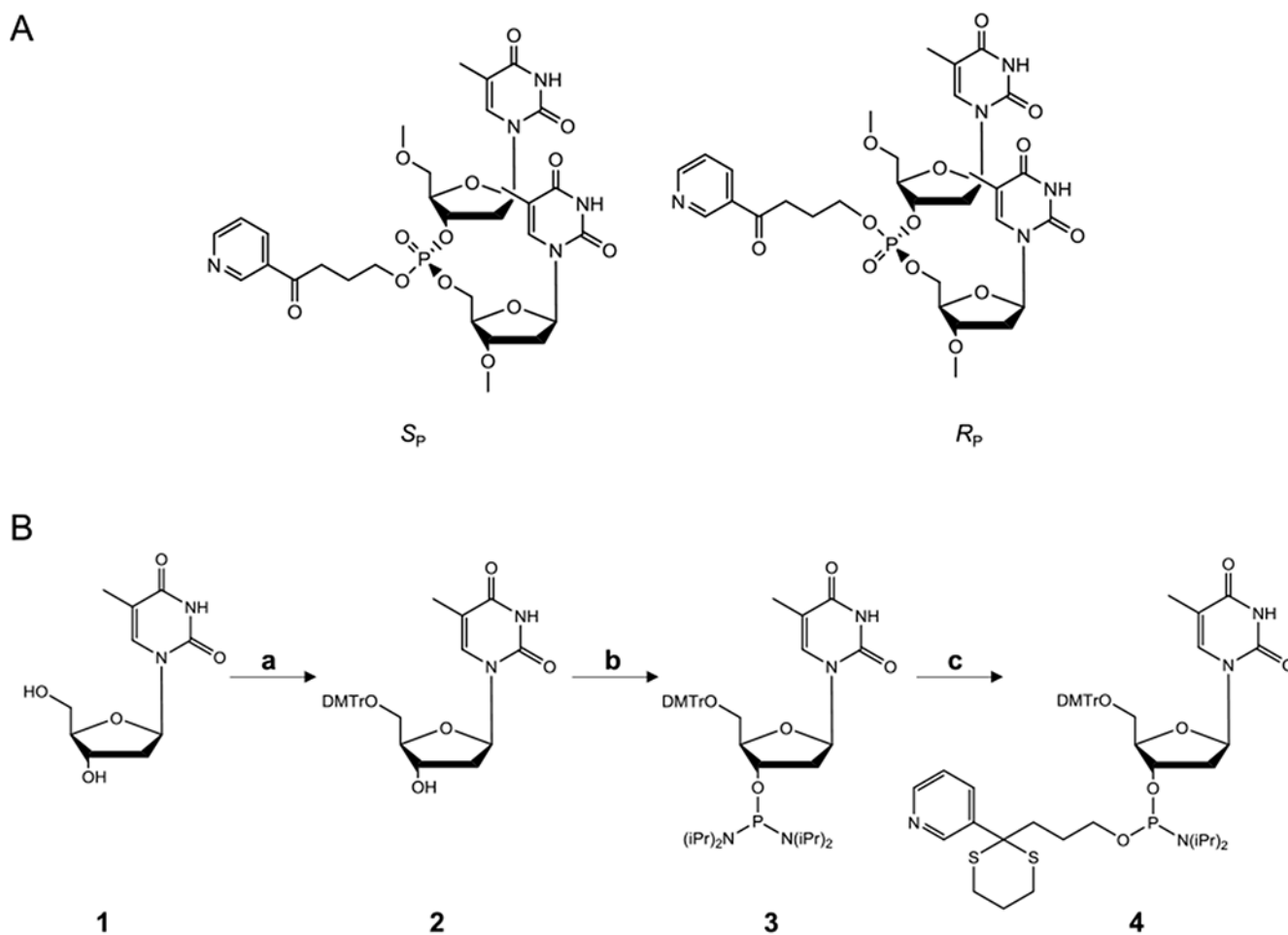
Scheme 1. (A) Structures of S_P and R_P Diastereomers of POB-PTEs at TT Site, (B) Chemical Synthesis of POB Phosphoramidite Building Block[1]

[1]Reagents and conditions: (a) 4,4′-dimethoxytrityl chloride, 4-dimethylaminopyridine, pyridine, R.T., 10 h; (b) bis-(diisopropylamino)chlorophosphine, *N,N*-diisopropylethylamine, dichloromethane, R.T. 1 h, (c) 4-(1,3-dithian-2-yl)-4-(3-pyridyl)butanol, ethylthiotetrazole, acetonitrile, dichloromethane, R.T. 1 h.