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β,γ-CHF- and β,γ-CHCl-dGTP Diastereomers: Synthesis, Discrete ^{31}P NMR Signatures, and Absolute Configurations of New Stereochemical Probes for DNA Polymerases

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Supporting Information

ABSTRACT: Deoxynucleoside 5′-triphosphate analogues in which the β,γ-bridging oxygen has been replaced with a CXY group are useful chemical probes to investigate DNA polymerase catalytic and base-selection mechanisms. A limitation of such probes has been that conventional synthetic methods generate a mixture of diastereomers when the bridging carbon substitution is nonequivalent (X $\neq$ Y). We report here a general solution to this long-standing problem with four examples of β,γ-CXY dNTP diastereomers: (*S*)- and (*R*)-β,γ-CHCl-dGTP (**12a-1**/**12a-2**) and (*S*)- and (*R*)-β,γ-CHF-dGTP (**12b-1**/**12b-2**). Central to their preparation was conversion of the prochiral parent bisphosphonic acids to the P,C-dimorpholinamide derivatives **7** of their (*R*)-mandelic acid monoesters, which provided access to the individual diastereomers **7a-1**, **7a-2**, **7b-1**, and **7b-2** by preparative HPLC. Selective acidic hydrolysis of the P−N bond then afforded "portal" diastereomers, which were readily coupled to morpholine-activated dGMP. Removal of the chiral auxiliary by H_2 (Pd/C) gave the four individual diastereomeric nucleotides **12**, which were characterized by ^{31}P, ^{1}H, and ^{19}F NMR spectroscopy and by mass spectrometry. After treatment with Chelex-100 to remove traces of paramagnetic ions, at pH ~10 the diastereomer pairs **12a,b** exhibit discrete P_α and P_β ^{31}P resonances. The more upfield P_α and more downfield P_β resonances (and also the more upfield ^{19}F NMR resonance in **12b**) are assigned to the *R* configuration at the P_β-CHX-P_γ carbons on the basis of the absolute configurations of the individual diastereomers as determined from the X-ray crystallographic structures of their ternary complexes with DNA and polymerase β.

Nucleotide bisphosphonate analogues in which a pyrophosphate bridging oxygen is replaced by a methylene carbon were first described by Myers.[1] Subsequently, it was suggested that the bisphosphonate moiety more closely mimics pyrophosphate when the bridging carbon is fluorinated.[2] P_α-CXY-P_β substitution blocks nucleotidyl transfer catalyzed by polymerases, whereas P_β-CXY-P_γ substitution (Figure 1) produces a dNTP substrate analogue with leaving-group properties that can be tuned by the substituents. The conventional syntheses of α,β- and β,γ-CXY nucleotide analogues involve coupling of bisphosphonates with nucleosides and nucleoside monophosphates, respectively.[3] The α,β- and β,γ-analogues where X $\neq$ Y are therefore obtained as mixtures of two diastereomers because a new chiral center is generated at the bisphosphonate bridging carbon (Figure 1). Several β,γ-CXY nucleotide analogues have been investigated as enzymatic probes for DNA, viral RNA, or RNA-directed DNA polymerases,[4] but the potential for a stereospecific interaction of these diastereomeric analogues with the enzyme active site has received little attention until recently.[5]

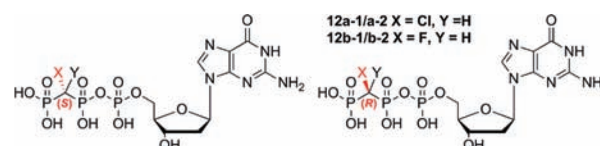

Figure 1. Diastereomeric β,γ-CXY analogues of dGTP.

DNA polymerase β (pol β) is a key polymerase in short-patch base excision repair (BER).[6] This is the predominant BER pathway in humans and is crucial for maintaining genome integrity. Pol β has been an extensively studied model for understanding polymerase fidelity.[6c,7] In a recent study of the thus-far obscure transition state of pol β, we reported a series of α,β- and β,γ-CXY dNTP analogues as structural, functional, and fidelity probes.[8] The use of a systematically varied β,γ-CXY group to probe for a leaving-group effect in the catalytic process provided evidence for a base-match-dependent chemical transition step,[8b,c] consistent with the findings of Lin et al.[9] and Tsai et al.[10] using different approaches.

X-ray crystallographic studies of ternary complexes formed from diastereomeric β,γ-CXY dGTP analogue mixtures incubated with binary DNA-pol β complex crystals have revealed the presence of only one diastereomer in the active site for monofluorinated analogues (X = H, Cl, Me; Y = F), associated with an interaction between the F atom and Arg183.[5,11] Other β,γ-monohalogenated (X = H; Y = Cl, Br), monomethylated (X = H, Y = Me), and heterodihalogenated

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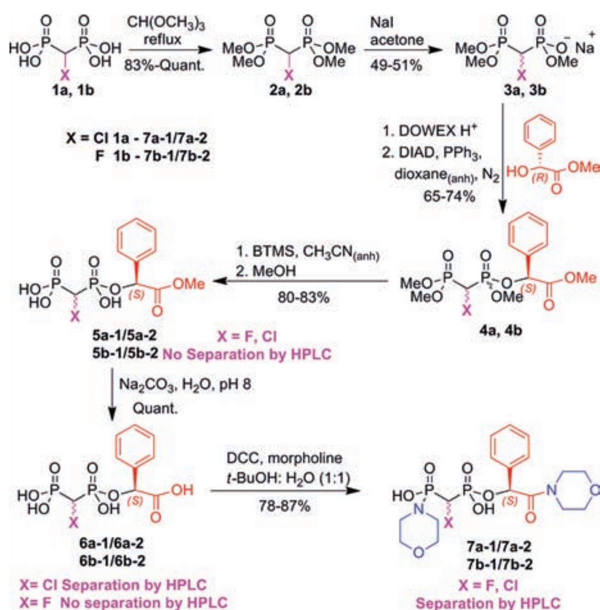
(X = Cl, Y = Br) analogues populated the active site evenly in the crystal complex.[5] These observations provided a strong impetus to obtain the individual diastereomers.

Nucleotide-analogue stereoisomers resulting from replacing a nonbridging P_α or P_β oxygen with B, S, or Se have been isolated by HPLC, or, in the case of ATPαS and ATPβS, by selective enzymatic depletion.[12] In contrast, separation of diastereomers where a bridging oxygen is replaced by a CXY group has remained a long-standing challenge. The diastereomers of α,β-CMe(N_3)-dATP were recently prepared via the corresponding dADP isomers, which could be separated by preparative reversed-phase C_{18} HPLC.[13] However, efforts to separate the α,β-CH(N_3) stereoisomers were unsuccessful.[13] (R/S)-β,γ-CH(N_3)-dGTP and (R/S)-β,γ-CMe(N_3)-dGTP also proved refractory to this method,[13] suggesting that both the substituent size and the distance of the CXY group from the chiral ribose affect separation. It seemed apparent that preparation of the elusive individual β,γ-CXY stereoisomers, particularly the highly desirable monohalo derivatives,[5,14] required a new approach, ideally one achieving stereochemical separation at the bisphosphonate level.

Here we report the synthesis, analytically discrete ^{31}P NMR signatures, and absolute configuration assignments of all four diastereomers of β,γ-CHX-dGTP (X = F, Cl), based on fixing the chirality at the bridging carbon of the prochiral α-halo bisphosphonate prior to conjugation using a novel chiral auxiliary strategy. After separation, the intermediate is conjugated conventionally with the targeted dNMP. As the incorporated nucleoside suffices to maintain the bisphosphonate stereochemistry, the chiral auxiliary can then be removed reductively under mild, nonracemizing conditions.

Scheme 1. Synthesis of Chiral Bisphosphonate Synthons 7



Synthesis of the chiral bisphosphonate synthons **7a-1**/**7a-2** and **7b-1**/**7b-2** is outlined in Scheme 1. The readily available α-halo bisphosphonic acid **1a**[15] or **1b**[2a,5a,16] was heated at reflux with trimethylorthoformate to afford the corresponding tetramethyl ester **2a**[17] or **2b**.[18] Monodemethylation using 1 equiv of NaI in acetone[19] afforded the racemic trimethyl esters **3**, which were converted to their acid forms on Dowex (H$^+$) and then esterified with (R)-(−)-methyl mandelate using Mitsunobu coupling, giving esters **4** with inversion at the chiral center of the auxiliary.[20] These mixtures of diastereomers were subjected to selective silyldemethylation with BTMS[21] in anhydrous CH_3CN followed by methanolysis to afford the (S)-mandelyl bisphosphonates **5a,b** as diastereomer pairs.

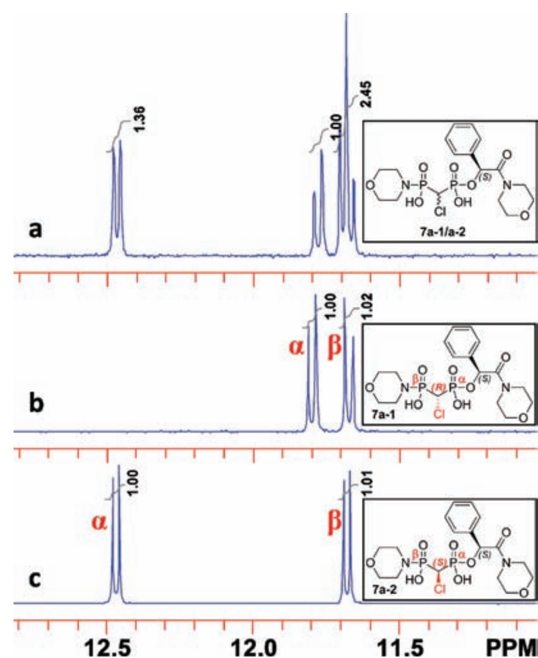
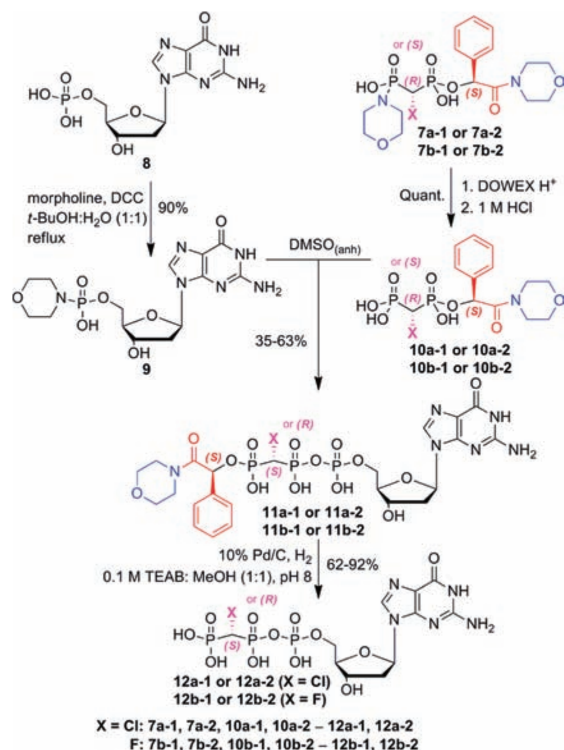


Figure 2. ^{31}P NMR spectra (202 MHz, D_2O) of (a) a diastereomeric mixture of **7a-1**/**7a-2** at pH 9.8, (b) diastereomer **7a-1** at pH 9.8, and (c) diastereomer **7a-2** at pH 10.0.

Attempts to separate the stereoisomers of **5** by RP-C_{18} HPLC were not successful. However, after facile (pH 8) hydrolysis of the carboxylate methyl ester (which proceeded without loss of stereochemistry at the benzyl carbon), the resulting mandelic acid bisphosphonate esters **6a-1**/**6a-2** could be separated by preparative RP-C_{18} HPLC under isocratic conditions. Unfortunately, the α-fluoro bisphosphonate diastereomers **6b-1**/**6b-2** could not be resolved by this method, even on analytical scale. To explore the effect of masking both the carboxylic and phosphonic acid groups on improving the chromatographic separability, we converted the mixtures of diastereomers of **6** to the corresponding P,C-dimorpholinamides **7**, which gratifyingly made possible the facile preparative isolation of all four diastereomers **7a-1**, **7a-2**, **7b-1**, and **7b-2** by preparative HPLC. The ^{31}P NMR spectra of **7a** before and after separation are shown in Figure 2. The relatively more downfield chemical shift in each diastereomer corresponds to the benzyl ester phosphonate P nucleus, based on the ^{1}H−^{31}P gradient heteronuclear multiple bond correlation between the downfield phosphorus peak and the benzyl proton.

Formation of the target nucleotides was carried out by conjugation with a 5′-activated dGMP (Scheme 2). The isolated **7a** or **7b** stereoisomer was first exchanged on a Dowex H$^+$ column, and the pH of the eluate was adjusted to 1 (1 M HCl) to complete the hydrolysis of the P−N bond, giving the "portal" monoesters **10**. Each of these diastereomers was coupled with dGMP-morpholidate (**9**)[5] [prepared by N,N-dicyclohexylcarbodiimide coupling of dGMP (**8**) with morpholine][22] by stirring in anhydrous DMSO for 3 days to afford the

Scheme 2. Synthesis of Target Diastereomeric Nucleotides



nucleoside triphosphate analogues **11**. These were purified by strong anion exchange HPLC and obtained as the triethylammonium salts. Removal of the chiral morpholinamide auxiliary by hydrogenolysis over 10 wt% Pd/C in 0.1 M triethylammonium bicarbonate/MeOH (1:1, pH 8) gave the deprotected individual β,γ-CHCl-dGTP (**12a-1**/**12a-2**) and β,γ-CHF-dGTP (**12b-1**/**12b-2**) diastereomers, which were then purified by RP-C_{18} HPLC and obtained as the triethylammonium salts.

The ^{19}F NMR spectrum of the **12b-1**/**12b-2** mixture as obtained by conventional synthesis was previously reported to display nonoverlapping peaks for the two diastereomers.[5a,b] The chemical shifts and correct coupling constants of the two

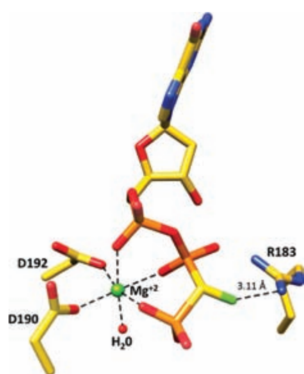


Figure 3. Detailed view of the incoming nucleotide (*R*)-β,γ-CHF-dGTP (**12b-2**) in the active site of the X-ray crystal structure of its ternary complex with pol β and DNA (PDB entry 4DO9). The Arg183, Asp190, and Asp192 side chains in the enzyme active site are shown, along with the nucleotide-binding magnesium and a water molecule. The interatomic distance between the F atom and Nη2 of Arg183 is 3.11 Å.

Table 1. Relative ^{31}P and ^{19}F NMR Chemical Shifts and Absolute Configurations of β,γ-CHX-dGTP Diastereomer Pairs in D_2O[a]

Compound	^{31}P NMR P_α	^{31}P NMR P_β	^{19}F NMR	CHX A.C.
12a-1 (CHCl)	D	U	N/A	*S*
12a-2 (CHCl)	U	D	N/A	*R*
12b-1 (CHF)	D	U	D	*S*
12b-2 (CHF)	U	D	U	*R*

[a]Abbreviations: A.C., absolute configuration; D, downfield; U, upfield. NMR data: **12a-1**/**12a-2**: P_α, $\Delta\delta$ = 5.4 Hz; P_β, $\Delta\delta$ = 8.5 Hz (202 MHz, pH 10.2). **12b-1**/**12b-2**: P_α, $\Delta\delta$ = 2.4 Hz; P_β, $\Delta\delta$ = 5.3 Hz (162 MHz, pH 10.5); ^{19}F, $\Delta\delta$ = 22.6 Hz (376 MHz, pH 10.5). $\Delta\delta$ values were measured from the NMR spectra of the artificial mixtures.

diastereomers were derived by simulation[5b] but could not be assigned to a specific configuration at the β,γ-bridging carbon. The absolute configuration at the chiral CHF carbon of **12b-2** is found to be *R* by X-ray crystallographic analysis of its ternary complex with DNA and pol β (Figure 3; PDB entry 4DO9), allowing the assignment of the more upfield ^{19}F resonance to this diastereomer (Table 1). It was possible to obtain the absolute configurations of the other three diastereomers (**12b-1**, **12a-1**, and **12a-2**) similarly (PDB entries 4DOA, 4DOC, and 4DOB, respectively).

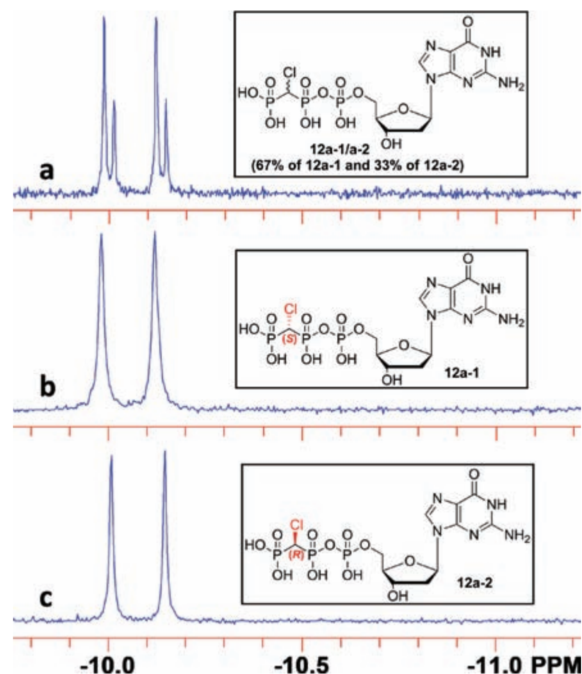


Figure 4. ^{31}P NMR spectra (202 MHz, D_2O) of P_α in **12a**: (a) Artificial mixture of **12a-1**/**12a-2** at pH 10.2. **12a-1** was added in excess, demonstrating that the **12a-2** signal is more upfield (U in Table 1) by $\Delta\delta$ = 5.4 Hz (0.027 ppm). (b) Diastereomer **12a-1** at pH 10.6. (c) Diastereomer **12a-2** at pH 10.3.

The ability to detect discrete ^{31}P resonances for non-F-containing analogues such as **12a-1**/**12a-2** would render them more generally useful as stereoprobes. At pH ~10 and after removal of traces of paramagnetic metal ions by passage through Chelex-100, the diastereomeric P_α and P_β resonances of **12a-1**/**12a-2** and **12b-1**/**12b-2** proved to be observable at 162 and 202 MHz (0.30 Hz/point digital resolution). As shown in Figure 4, a 2:1 mixture of **12a-1**/**12a-2** exhibits a ^{31}P $\Delta\delta$ of

5.4 Hz for P_α at 202 MHz, leading to the assignment of the more downfield signal to the *S* isomer, **12a-1**. The P_β resonances, which are separated by 8.5 Hz under the same conditions, show the reverse relationship (Table 1).

In conclusion, the first examples of individual β,γ-CXY-dNTP diastereomers have been successfully prepared, and their absolute configurations have been correlated with discrete features of their ^{31}P and ^{19}F NMR spectra. The synthetic strategy developed, based on constructing a "portal" chiral bisphosphonate synthon, should be adaptable to the synthesis of cognate nucleotide bisphosphonate diastereomers. The availability of the individual diastereomers of **12a,b** now makes possible kinetic analysis of their binding and turnover interactions with pol β and other polymerases.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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