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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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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 non-equivalent (X ≠ Y). We report here a general solution to this long-standing problem with four examples of individual *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 achiral parent bisphosphonic acids to P,C-dimorpholinamide derivatives (**7**) of their (*R*)-mandelic acid monoesters (**6**), 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 the " portal " diastereomers **10**, which were readily coupled to morpholine-activated dGMP. Removal of the chiral auxiliary by H_2 (Pd/C) afforded the four individual diastereomeric nucleotides (**12**), which were characterized by ^{31}P, ^{1}H and ^{19}F NMR, and by MS. After treatment with Chelex®-100 to remove traces of paramagnetic ions, at pH ~10 the diastereomer pairs **12a** and **12b** 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, based on the absolute configurations of the individual diastereomers as determined by X-ray crystallographic structures of their ternary complexes with DNA-pol *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 will block nucleotidyl transfer catalyzed by polymerases, whereas P_β-CXY-P_γ substitution (Fig. 1) produces a dNTP substrate analogue with leaving group properties that can be tuned by the substituents. Conventional synthesis of *α,β*- or *β,γ*-CXY nucleotide analogues involves coupling of bisphosphonates with

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SUPPORTING INFORMATION

Details of synthesis and characterization data for **2-7** and **10-12**, NMR spectra of synthetic (**6a**, **7** and **12**) and artificial (**12**) mixtures, Chelex®-100 effect on resolution of **12a** ^{31}P NMR, crystal structures of **12a-1**, **12a-2** and **12b-1**, crystallographic data for **12b-2**, HPLC conditions (**6a**, **7**, **11** and **12**). This information is available free of charge via the Internet at http://pubs.acs.org.

nucleosides or nucleoside monophos-phates, respectively.[2c,2d] The α,β- and β,γ-analogues where X ≠ Y are therefore obtained as mixtures of two diastereomers due to the generation of a new chiral center at the bisphosphonate bridging carbon (Fig. 1). Several β,γ-CXY nucleotide analogues have been investigated as enzymatic probes for DNA, viral RNA or RNA-directed DNA polymerases,[3] but the potential for a stereospecific interaction of these diastereomeric analogues with the enzyme active site has received little attention[3c-g,3i] until recently.[4,5]

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 revealed a base match-dependent chemical transition step,[8b,8c] 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 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 fluorine atom and Arg183.[4,11] Other β,γ-monohalogenated (X = H; Y = Cl, Br), monomethylated (X = H, Y = Me) and heterodihalogenated (X = Cl, Y = Br) analogues populated the active site evenly in the crystal complex.[4] These observations provided a strong impetus to obtain the individual diastereomers.

Nucleotide analogue stereoisomers resulting from replacing a non-bridging P_α or P_β oxygen with boron, sulfur or selenium have been isolated by HPLC, or, in the case of ATPαS and ATPβS, by selective enzymatic depletion.[12] In contrast, diastereomers where a bridging oxygen is replaced by a CXY group have proven elusive. The diastereomers of α,β-CMe(N_3) dATP were recently prepared via the corresponding dADP isomers, which could be separated by preparative RP-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) 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 CXY group from the chiral ribose affects separation. It seemed apparent that preparation of the elusive individual β,γ-CXY stereoisomers, particularly the highly desirable monohalo derivatives,[4-5,14] required a new approach, ideally one achieving stereochemical separation at the bisphosphonate level.

Here, we communicate the synthesis, analytically discrete ^{31}P NMR signatures, and absolute configuration assignments of all four diastereomers of β,γ-CHX dGTP (X = F or 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, unracemizing conditions.

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,4a,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 afforded the racemic trimethyl esters **3**,[19] 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 by BTMS[21] in anhy-drous CH_3CN followed by methanolysis[21] to afford the (*S*)-mandelyl bisphosphonates (**5a** or **5b**) as diastereomer pairs.

Attempts to separate the **5** stereoisomers by RP-C_{18} HPLC were not successful. However, the diastereomers 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** and **6a-2** could be separated on preparative RP-C_{18} HPLC under isocratic condition. Unfortunately, the α-fluoro bisphosphonate diastereomers **6b-1** and **6b-2** could not be resolved suitably by this method, even on analytical scale. Reasoning that masking both the carboxylic and the phosphonic acid groups might improve chromatographic separability, we converted the **6** diastereomer mixtures to the corresponding P,C-dimorpholinamides (**7**), which gratifyingly made possible facile preparative isolation of all four individual diasteromers **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 Fig. 2. The relatively more downfield chemical shift in each individual diastereomer corresponds to the benzyl ester phosphonate P nucleus, based on ^{1}H-^{31}P gHMBC (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** stereoisomers were first exchanged on a Dowex H^+ column and the pH of the eluate was adjusted to 1 (1 M HCl) to complete hydrolysis of the P-N bond, giving the “portal” monoesters (**10**). Each of these diastereomers was coupled with dGMP-morpholidate (**9**)[4a] (prepared by DCC coupling of dGMP, **8**, with morpholine)[22] by stirring in anhydrous DMSO for 3 d to afford the nucleoside triphosphate analogues **11**. These were purified by strong anion exchange (SAX) HPLC and obtained as triethylammonium salts.[4a] Removal of the (*R*)-mandelic acid morpholinamide auxiliary by hydrogenolysis over 10 wt. % Pd/C in 0.1 M TEAB:MeOH (1:1, pH 8) gave the deprotected individual dGTP β,γ-CHCl (**12a-1**, **12a-2**) and β,γ-CHF (**12b-1**, **12b-2**) diastereomers, which were then purified by RP-C_{18} HPLC and obtained as triethylammonium salts.

The ^{19}F NMR spectrum of the **12b-1/12b-2** mixture as obtained by conventional synthesis was previously reported to display non-overlapping peaks for the two diastereomers.[4a] The chemical shifts and correct coupling constants of the two diastereomers were derived by simulation,[4b] 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-pol β (Fig. 3, PDB ID: 4DO9), allowing 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 individual diastereomers (**12b-1**, **12a-1** and **12a-2**) similarly (PDB IDs: 4DOA, 4DOC and 4DOB, respectively).

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 removing traces of paramagnetic metal ions by passage through Chelex®-100, the individual diastereomeric P_α and P_β resonances of **12a-1/12a-2** and **12b-1/12b-2** proved to be observable at both 162 and 202 MHz (0.30 Hz/point digital resolution). As shown in Fig. 4, a 2:1 mixture of **12a-1** and **12a-2** exhibits a ^{31}P $\Delta\delta$ of 5.4 Hz for P_α at 202 MHz and assigns the more downfield signal to the (*S*) isomer, **12a-1**. The P_β resonances, which are separated by 8.5 Hz under same conditions, show the reverse relationship (Table 1).

In conclusion, the first examples (**12**) 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 a common chiral bisphosphonate synthon, should be adaptable to the synthesis of cognate nucleotide bisphosphonate diastereomers. The availability of the individual diastereomers of **12a** and **12b** now makes possible kinetic analysis of their individual binding and turnover interactions with pol β and other polymerases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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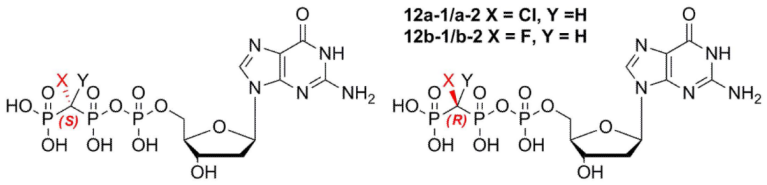


Figure 1.
Diastereomeric β,γ-CXY analogues of dGTP.

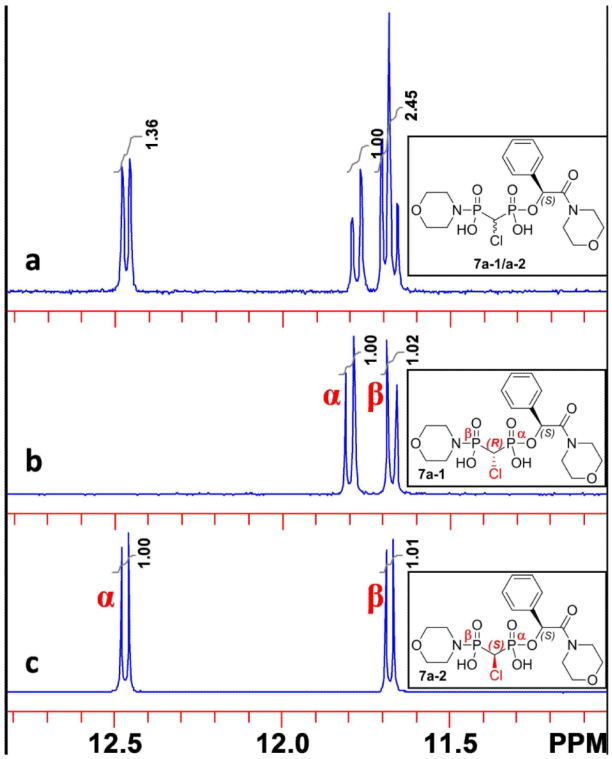


Figure 2.

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

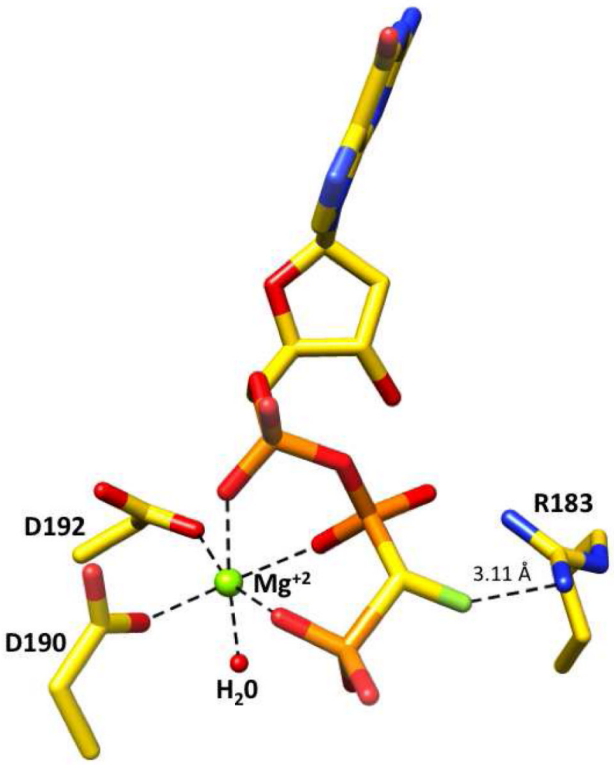


Figure 3.
Detailed view of the incoming nucleotide **12b-2**, (*R*)-*β,γ*-CHF dGTP in the active site of the X-ray crystal structure of its ternary DNA pol *β*:DNA (PDB ID: 4DO9). The enzyme active site Arg183, Asp190 and Asp192 side chains are shown, along with the nucleotide-binding magnesium and a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

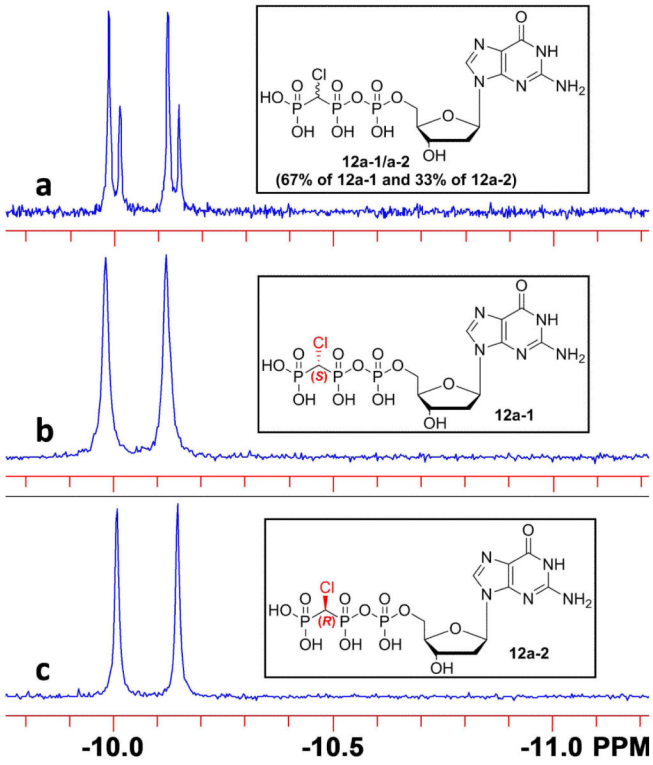
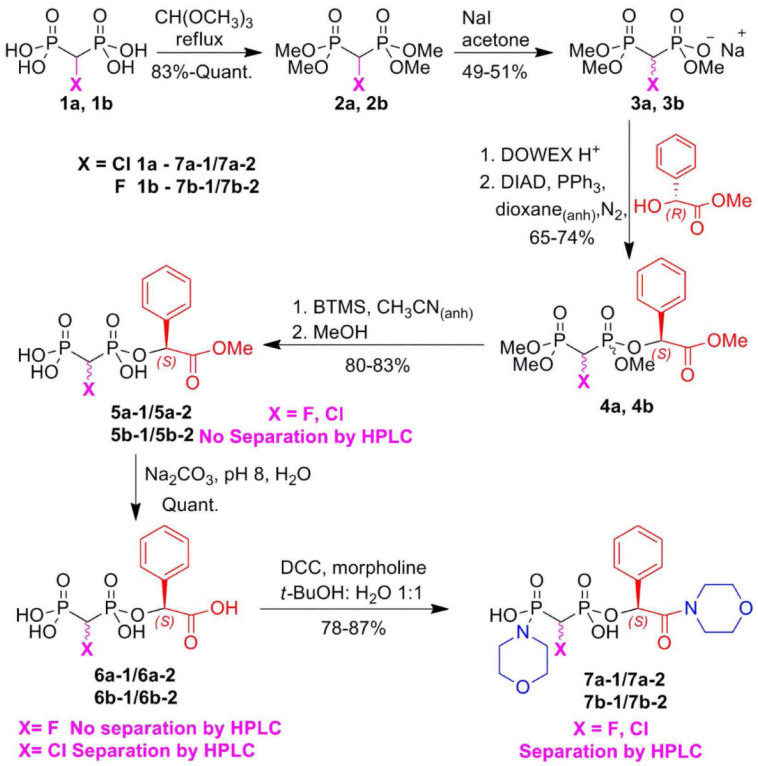
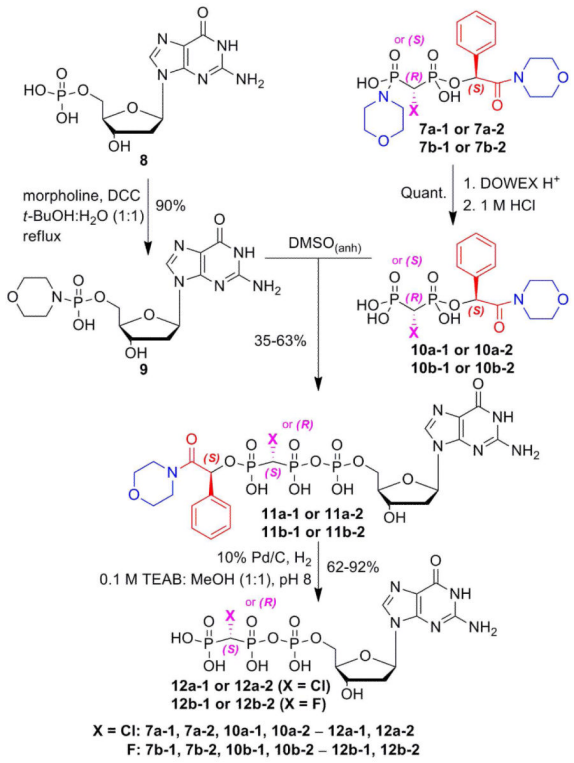


Figure 4.

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



Scheme 1.
Synthesis of the chiral bisphosphonate synthons 7.



Scheme 2.
Synthesis of target diastereomeric nucleotides.

Table 1

Relative ^{31}P and ^{19}F NMR chemical shifts and absolute configurations of β,γ-CHX dGTP diastereomer pairs

Compounds	^{31}P NMR P_α	^{31}P NMR P_β	^{19}F NMR	A.C.[a] CHX
12a-1 (CHCl)	D[b]	U[c]	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] A.C. = absolute configuration;

[b] D = downfield;

[c] U = upfield. **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 are measured from the NMRs of the artificial mixtures.