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Completing the β,γ-CXY-dNTP Stereochemical Probe Toolkit: Synthetic Access to the dCTP Diastereomers and ^{31}P and ^{19}F NMR Correlations with Absolute Configurations

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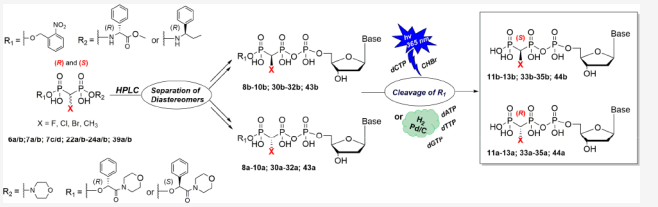
ABSTRACT: Nucleoside 5′-triphosphate (dNTP) analogues in which the β,γ-oxygen is mimicked by a CXY group (β,γ-CXY-dNTPs) have provided information about DNA polymerase catalysis and fidelity. Definition of CXY stereochemistry is important to elucidate precise binding modes. We previously reported the (*R*)- and (*S*)-β,γ-CHX-dGTP diastereomers (X = F, Cl), prepared via P,C-dimorpholinamide CHCl (**6a**, **6b**) and CHF (**7a**, **7b**) bisphosphonates (BPs) equipped with an (*R*)-mandelic acid as a chiral auxiliary, with final deprotection using H_2/Pd. This method also affords the β,γ-CHCl-dTTP (**11a**, **11b**), β,γ-CHF (**12a**, **12b**), and β,γ-CHCl (**13a**, **13b**) dATP diastereomers as documented here, but the reductive deprotection step is not compatible with dCTP or the bromo substituent in β,γ-CHBr-dNTP analogues. To complete assembly of the toolkit, we describe an alternative synthetic strategy featuring ethylbenzylamine or phenylglycine-derived chiral BP synthons incorporating a photolabile protecting group. After acid-catalyzed removal of the (*R*)-(+)-α-ethylbenzylamine auxiliary, coupling with activated dCMP and photochemical deprotection, the individual diastereomers of β,γ-CHBr- (**33a**, **33b**), β,γ-CHCl- (**34a**, **34b**), β,γ-CHF-dCTP (**35a**, **35b**) were obtained. The β,γ-CH(CH_3)-dATPs (**44a**, **44b**) were obtained using a methyl (*R*)-(−)-phenylglycinate auxiliary. ^{31}P and ^{19}F NMR Δδ values are correlated with CXY stereochemistry and pK_{a2-4} values for 13 CXY-bisphosphonic acids and imidodiphosphonic acid are tabulated.



INTRODUCTION

Nucleoside triphosphates play critical roles in innumerable aspects of biology and medicine.[1,2] High fidelity in DNA replication is essential to maintain the integrity of the genome and avoid mutations which may lead to human diseases such as cancer.[2−9] The molecular interactions and processes underlying the catalytic efficiency and exquisite base-specific selectivity of DNA polymerases have therefore been a continuing focus of intense study.[10−19] Nucleotide analogues have proven to be essential tools in the effort to identify intermediate structures and individual steps in the mechanisms of different DNA polymerases.[20−25]

We previously reported[26−30] deoxynucleoside 5′-triphosphate bisphosphonate analogues in which the β,γ-bridging oxygen of the triphosphate moiety (dNP$_\alpha$-O-P$_\beta$-O-P$_\gamma$) is replaced by a substituted methylene group (CXY) as probes of the ground state (GS) and the transition state (TS) in pol β-catalyzed DNA repair, including by the cancer-associated K289M mutant of pol β.[29,31] In addition to β,γ-CXY-dNTPs (N = G, T, and most recently[29] C and A), β,γ-imido-dNTP (β,γ-NH-dNTP, N = A and G) analogues have also been used to study the mechanism of DNA polymerization catalyzed by pol η, which is implicated in the prevention of skin cancer by copying past cyclobutene dimers in UV-damaged DNA.[32] This approach has been extended to unnatural nucleoside triphosphates (β,γ-CF_2-dNaMTP and β,γ-CF_2-dTPT3TP) as a strategy to avoid degradation by cellular and secreted phosphatases after cellular uptake.[33,34]

These β,γ-modified dNTP analogues (Figure 1) mimic natural dNTP substrates of DNA polymerases in enabling template-dependent incorporation of dNMP into DNA primer, but their complexes with the enzymes can exhibit different ground-state dissociation constants (K_d) and turnover rate constants (k_{pol}) due to their modified triphosphate groups. The latter case will be expected for a rate-determining step corresponding to a transition state sensitive to triphosphate charge stabilization. This should be the case in a "chemical" rate-determining catalytic step in which the pyrophosphate (PPi) leaving group acquires a negative charge as a result of attack of the DNA primer strand terminal 3′-OH oxygen on P$_\alpha$

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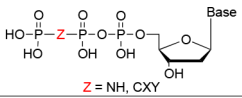

Base	Z	Individual Diastereomers Z
G	NH, CH_2, CHF, CHCl, CHBr, $CHCH_3$, CHN_3, CF_2, CCl_2, CBr_2, $C(CH_3)_2$, CFCl, CFBr, $CFCH_3$, CN_3CH_3	CHF, CHCl
T	CH_2, CHF, CHCl, CHBr, $CHCH_3$, CF_2, CCl_2, CBr_2, CFCl	CHCl
A	NH, CH_2, CHF, CHCl, $CHCH_3$, CF_2, CCl_2, CBr_2, CFCl	$CHCH_3$, CHF, CHCl
C	CH_2, CHF, CHCl, CHBr, CF_2, CCl_2, CBr_2, CFCl,	CHF, CHCl, CHBr

Figure 1. β,γ-Z-dNTP (N = G,[26,32,35−38] T,[27] C,[27,29] A[32]).

of the incoming dNTP or analogue with P_α−OP_β bond cleavage. While relative nucleoside triphosphate charge in the transition state cannot be directly accessed experimentally, it can be approximated by the pK_{a4} of the pyrophosphate or bisphosphonate leaving group. Systematic variation of X and Y in α,α-substituted methylenebis(phosphonic acids)[39] (Z = CXY) using hydrogen, halo, azido,[38] or methyl substituents provides a broad range of pK_{a4} values bracketing the pK_{a4} of PPi (8.9), from 7.8 for CF_2 to 12.3 for $C(CH_3)_2$, permitting the construction of linear free energy (LFER) plots of log k_{pol} vs pK_{a4} to investigate leaving group effects on the kinetics of DNA polymerization reactions (Figure 2).[26,27,29,32,40]

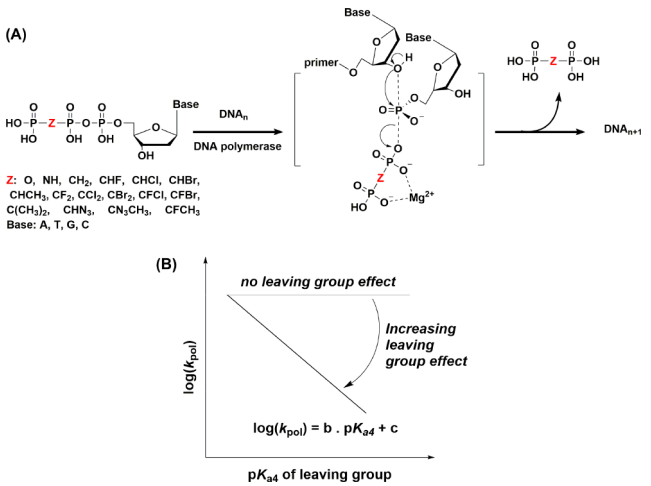

Figure 2. Using β,γ-Z-dNTPs (k_{pol} value data and bisphosphonate leaving group pK_{a4} data) for LFER construction. (A) Chemical step of β,γ-Z-dNTP incorporation into the daughter DNA strand (additional active site interactions, e.g., to stabilize negative charge on the P_α are omitted). (B) Ideal LFER Brønsted plot of log(k_{pol}) vs pK_{a4} for no leaving group effect (b = 0, chemical step not rate-determining) and significant leaving group effect (b < 0, chemical step is rate-determining).

Structural and enzyme kinetic data have revealed significant dependence of pol LFER signatures on the nucleotide base progressing from dGTP[26,32,35−38] to dTTP[27] and more recently dATP[32] and dCTP[29,40] analogues, underlining the value of a base-diverse toolkit encompassing all four DNA bases to elucidate subtle (and not so subtle) fidelity-dependent effects on catalysis. An important consideration is the fact that in analogues where X ≠ Y the CXY moiety is chiral, resulting in diastereomer pairs (Figure 3) which may be accommodated

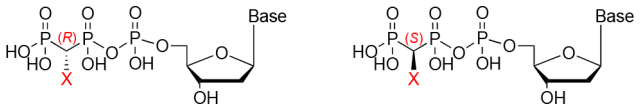

Figure 3. Individual β,γ-CHX-dNTP diastereomers. (N = G: introduced by Wu et al.;[35] N = C, A, T: this work).

in the asymmetric active site of DNA polymerases with nonequivalent dissociation constants K_d and/or observed catalytic rate constants k_{pol}.[37] The individual isomers thus are intriguing probes of stereochemically defined differences in C−X/C−Y interactions with enzyme active-site residues, where otherwise the inherent acid−base properties of the two isomers would be expected to be similar. Other groups have reported synthesis of nucleoside triphosphates using a terminal triphosphate modifier[41] or the cyclic triphosphate route[42] without addressing the key issue of CXY stereochemistry.

We originally prepared the first examples of individual β,γ-CHX-dNTP diastereomers (N = G, X = F, Cl) using a chiral precursor, the (R)-mandelate of the appropriate bisphosphonic acid as a P,C-dimorpholinamide synthon (**6a**, **6b**, **7a**, **7b**; Figure 4A).[35] X-ray crystallographic structures of these

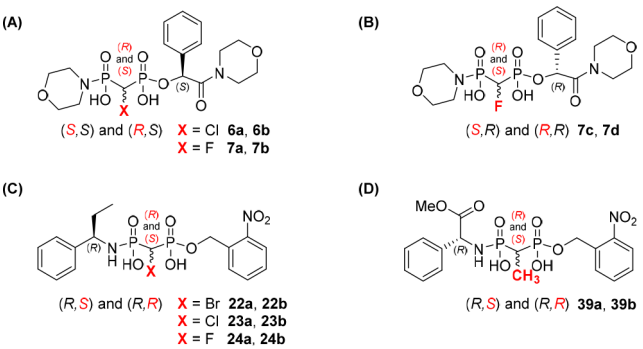

Figure 4. Achiral HPLC-separable chiral bisphosphonate synthons used for the preparation of individual (R)- and (S)-β,γ-CHX-dNTP. (A) Introduced by Wu et al.[35] (B−D) This work.

nucleotide analogues bound in ternary complexes with pol β/DNA established their absolute configurations, from which the CXY-configurations in the chiral precursors were trivially derived. The individual nucleotide diastereomers exhibited stereospecific differences in pol β binding and presteady-state kinetics.[35,37] ^{31}P and ^{19}F NMR studies of pol β turnover using both separate and mixed β,γ-CHF or β,γ-CHCl-dGTP diastereomer pairs as the dNTP substrates revealed that the (R)-CHX isomer was favored over the (S)-CHX isomer for G·C (correct) nucleotide incorporation into product DNA, with $[(k_{pol}/K_d)_R/(k_{pol}/K_d)_S]$ of 3.8 (F) and 6.3 (Cl).[43] Similarly, in the corresponding dATP analogues the (R)-isomer was favored over the (S)-isomer for correct incorporation of A opposite T by pol η with stereospecificities of 4.7 (F) and 4.8 (Cl) and by pol λ with a stereospecificity of 3.2 (F).[32] Thus, the orientation of C−X and C−Y within the active sites of these enzymes influences the energy of the transition state during turnover, and this was shown to be an even more pronounced effect for mispair incorporations.[32,43]

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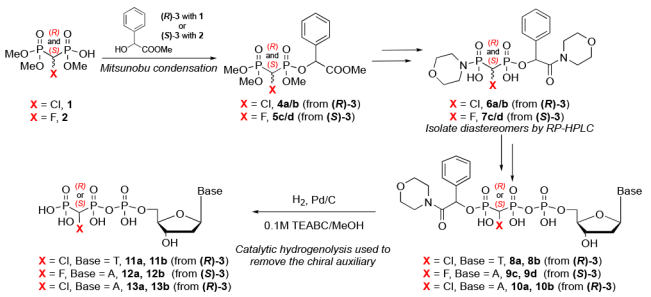
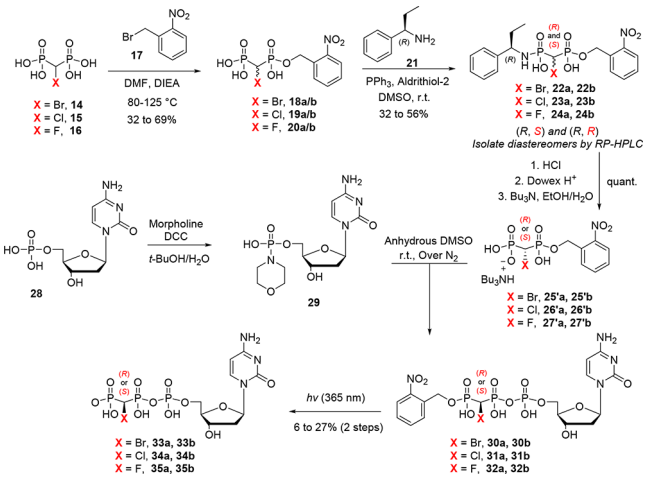
Although the (*R*)-mandelate bisphosphonic acid P,C-dimorpholinamide synthon also provides access to the individual *β,γ*-CHX-dATP (X = F, Cl) and *β,γ*-CHCl-dTTP diastereomers as detailed here (Scheme 1), it involves a

Scheme 1. Preparation of Individual Diastereomers of *β,γ*-CHCl-dTTP and *β,γ*-CHX-dATP (X = F, Cl) (11a, 11b, 12a, 12b, 13a, 13b)



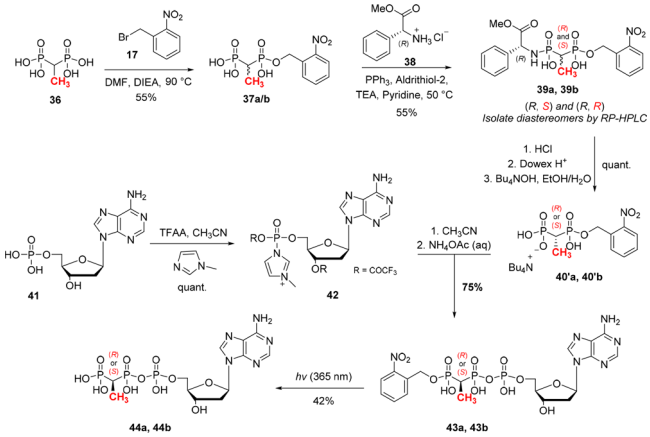
catalytic hydrogenation deprotection step that could be problematic with the cytosine heterocycle in CTP and also leads to reduction of the bromo substituent in *β,γ*-CHBr-dNTP analogues.[44,45] To complete assembly of a *β,γ*-CXY-dNTP toolkit encompassing all four DNA bases and including *β,γ*-CHBr-dNTPs, we report here an alternative synthetic route featuring the novel chiral BP synthons **22a**, **22b**, **23a**, **23b**, **24a**, and **24b** (Figure 4C). After acid-catalyzed removal of the (*R*)-(+)-*α*-ethylbenzylamine auxiliary, coupling with activated dCMP and photochemical deprotection, the individual *β,γ*-CHBr− (**33a**, **33b**), *β,γ*-CHCl− (**34a**, **34b**), and *β,γ*-CHF-dCTP (**35a**, **35b**) diastereomers (Scheme 2) are obtained in

Scheme 2. Synthesis of *β,γ*-CHX-dCTP (X = Br, Cl, F) Diastereomers (33a, 33b, 34a, 34b, 35a, 35b)



modest yield. The first examples of individual *β,γ*-CH(CH$_3$)-dNTP diastereomers **44a**, **44b**, which provide CHX substitution with different C−X polarity to the existing halo-substituted BP derivatives and extend the upper pK_{a4} range in LFER analyses, were also prepared by this new approach using methyl (*R*)-(−)-phenylglycinate (Figure 4D) as the chiral auxiliary (Scheme 3). The new methods constitute a more general and relatively facile stereospecific route to novel chiral bisphosphonate analogues of nucleotides that does not entail preparative chromatography requiring chiral separation media.

Scheme 3. Synthesis of *β,γ*-CHCH$_3$-dATP Diastereomers (44a, 44b)



Furthermore, analysis of ^{31}P NMR and ^{19}F NMR $\Delta\delta$ values between nucleotide diastereomer pairs are found to be predictive of absolute stereochemistry across the entire range of dNTP analogues examined. Finally, we include a self-consistent set of experimental pK_{a2-4} values for 13 *α,α*-substituted methylenebis(phosphonic acids) and imidodiphosphoric acid used to prepare our expanded set of nucleotide analogues for reference in constructing LFER plots (Figure 2).

RESULTS AND DISCUSSION

Desiderata and Design. The P,C-dimorpholinamide CHCl (**6a**, **6b**) (*S*)-mandelate and P,C-dimorpholinamide CHF (**7c**, **7d**) (*R*)-mandelate synthons (Figure 4A,B) were evaluated for suitability in preparing the corresponding individual *β,γ*-CHCl-dTTP and *β,γ*-CHX-dATP (X = F, Cl) diastereomers (Scheme 1). In our original route to the individual (*R*)- and (*S*)-*β,γ*-CHX-dGTP (X = F, Cl) diastereomers[35] we utilized (*R*)-methyl mandelate, (***R***)-**3**, to convert appropriate *α*-halomethylenebis(phosphonate) **1**, **2** into a mixture of the (*R,S*)- and (*S,S*)-P,C-dimorpholinamide bisphosphonate derivatives **6a**, **6b** and **7a**, **7b** (Figure 4A), which were easily separated by preparative HPLC using an achiral RP-C18 column (Scheme 1). Following regioselective acidic removal of the P-morpholinamide group and attachment to dGMP, the chiral auxiliary was removed by (Pd/C) catalytic hydrogenolysis to give the individual *β,γ*-CHX nucleotide analogues. As fully documented here (Scheme 1), this approach provides the individual diastereomers of the corresponding dTTP and dATP analogues (**11a**, **11b**, **12a**, **12b**, **13a**, **13b**). The triethylammonium content in the corresponding salts of the final dNTP analogues were 1−2.5 equiv, calculated from the ^{1}H NMR spectra after repeated coevaporation of the HPLC fractions with water under reduced pressure.

It should be noted that (*S*)-methyl mandelate, (***S***)-**3**, could be substituted for (***R***)-**3** in this synthesis but should reverse the HPLC elution order for the resulting chiral synthons (e.g., **7a**, **7b** and **7c**, **7d**) (Figure 4A,B). This was confirmed by using **7c**, **7d** (from **5c**/**d**) to prepare the individual *β,γ*-CHF-dATP diastereomers **12a**, **12b** (Scheme 1 and Table S1).

Although our original approach thus afforded the individual *β,γ*-CHF- and *β,γ*-CHCl-dGTP, dTTP, and dATP diastereomers, the (Pd/C) hydrogenolysis step to remove the mandelate auxiliary reduces the cytosine heterocycle.[44] This

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step also causes reduction of the bromo substituent in the CHBr bisphosphonate intermediate (even with a chloro CHCl bisphosphonate moiety, careful attention to reaction conditions is required to avoid partial reduction to the CH_2 bisphosphonate). This was a serious deficiency because of the unique nature of cytosine as a component of DNA and RNA. Cytosine is inherently unstable (mutagenic spontaneous or APOBEC cytosine deaminase-catalyzed deamination to uracil[46]), has synonymity with uracil as a third base codon, and is susceptible to methylation to 5-methylcytosine by DNA methyltransferases,[47] making it of particular importance as a missing component in our dNTP cohort of individual CHX analogues. At the same time, inaccessibility of the bromo analogues deprived us of a stereoelectronically valuable halo substituent probe, albeit the mixed CHBr diastereomers are readily obtainable.[26,27,29] In response, we have created a new BP synthon with (*R*)-(+)-α-ethylbenzylamine **21** as the chiral auxiliary (attached to the BP via a phosphoramide bond) and using a photochemically cleavable *o*-nitrobenzyl group as the bisphosphonate protecting group (**22a**, **22b**, **23a**, **23b**, **24a**, **24b**) (Figure 4C).

Synthesis of 33a, 33b, 34a, 34b, 35a, and 35b. The new route again starts from the appropriate α-halo methylenebis(phosphonic acid) **14**–**16**[48−50] as outlined in Scheme 2. After monoesterification with *o*-(bromomethyl)-nitrobenzene[51] **17**, giving **18a/b**, **19a/b**, **20a/b** in fair to moderate yield. Chiral auxiliary **21** was then installed on the unsubstituted side of the bisphosphonic acid derivative by reaction in the presence of PPh_3 and aldrithiol-2 in DMSO at rt. The resulting mixtures (∼1:1) of diastereomers **22a/b**, **23a/b**, **24a/b** (Figure 4C) were separated with baseline resolution on a standard semipreparative RP-C18 HPLC column (e.g., **24a**, **24b**, Figure 5). The chiral auxiliary was

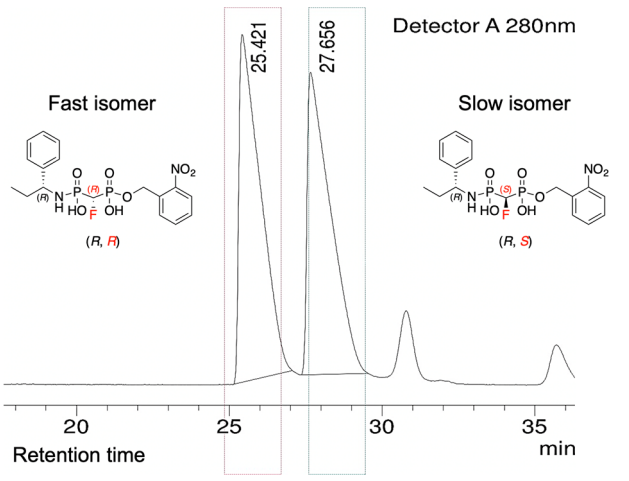

Figure 5. RP HPLC chromatogram: Phenomenex Luna 5 μm C18(2) 100 Å 21.2 mm × 250 mm column, 8.0 mL/min, 280 nm, isocratic mode, 27% acetonitrile in 0.1 M triethylammonium carbonate buffer at pH 8.5, loading 10 mg of crude **24a/b** mixture.

cleaved with aqueous HCl and passage through DOWEX H^+ to give **25a**, **25b**, **26a**, **26b**, **27a**, and **27b**. These intermediates were converted to the corresponding tributylammonium salts **25′a**, **25′b**, **26′a**, **26′b**, **27′a**, and **27′b** and conjugated via their free monophosphonic acid group with dCMP 5′-morpholidate[52] **29**, freshly prepared from deoxycytidine monophosphate **28**, and the final nucleotide analogues **33a**, **33b**, **34a**, **34b**, **35a**, and **35b** were obtained by photoirradiation at λ = 365 nm to remove the *o*-nitrobenzyl protecting group.[53,54] The final compounds were purified by dual-pass preparative HPLC (C18 and SAX) and obtained as triethylammonium salts (1.7−4 equiv, calculated from the ^1H NMR spectra).

Synthesis of 44a, 44b. To explore the robustness of our new approach, a different chiral auxiliary, methyl (*R*)-(−)-phenylglycinate[55] **38** and an alternative activated dAMP[56] **42** were used to obtain the individual β,γ-$CHCH_3$-dATP diastereomers **44a**, **44b**.

Following the procedure already described, the racemic mixture of monoesters **37a/b** was prepared and reacted with methyl (*R*)-(−)-phenylglycinate **38**, which gave superior results. The resulting (∼1:1) mixture of diastereomers **39a**, **39b** (Figure 4D) was separated on a RP HPLC column (Phenomenex Luna 5 μm C18(2) 100A 21.2 mm × 250 mm), loading up to 40 mg of the crude products (Experimental Section). After removal of the chiral auxiliary, the resulting intermediates **40′a** and **40′b** (in tetrabutylammonium salt format) were conjugated with 5′-dAMP-*N*-methylimidazolide[56] **42** (freshly prepared from **41**), and the final nucleotides **44a** and **44b** were obtained by photoirradiation at λ = 365 nm (Scheme 3).[53,54]

Stereochemistry. We previously demonstrated that mixtures of the β,γ-CHF and β,γ-CHCl-dGTP diastereomers display discrete ^{19}F and $^{31}P_{(\alpha,\beta)}$ NMR spectra,[35,37,57] which were confirmed for synthetic mixtures formed from the individual diastereomers and utilized to demonstrate that their turnover rate catalyzed by pol β during DNA synthesis is stereospecific for the CHX configuration.[35,36,43] The same stereochemical dependence was found in pre-steady-state enzyme kinetic experiments using the individual isomers.[26] Preferential binding of the more reactive stereoisomer from each CHX diastereomer pair was observed during the formation of ternary complexes with crystallized DNA-pol β and mapped to the more slowly eluting peak in preparative HPLC separation of the precursor bisphosphonate chiral synthons. This designated a defined stereospecific path to any individual β,γ-CHF- or β,γ-CHCl-dNTP analogue from each synthon. We determined the corresponding individual nucleotide analogue structures diffused into their pol β ternary complexes with template and gapped primer DNA by X-ray crystallography.[35−37] The crystal structures also revealed that the more reactive and better bound stereoisomer had the same (*R*)-configuration for both X = F and Cl, which placed the halogen atom in a position proximal to a guanidinium $N\eta^2$ of Arg183 in the active site of pol β (although this may not be the sole determining factor in stereoselection).[26]

The absolute configurations of the β,γ-CHCl-dNTP (N = T, A) stereoisomers (**11a**, **11b** and **13a**, **13b**), prepared using the same chiral BP synthon pair equipped with the (***R***)-**3** chiral auxiliary, were assigned similarly. This method was used for the CHF assignments of the corresponding dATP analogues (**12a**, **12b**) prepared using the (*S*)-methyl mandelate auxiliary (***S***)-**3**, and their stereopurity >99% was verified by their ^{19}F and ^{31}P NMR spectra.

In the case of the β,γ-CHX-dCTP (X = Br, Cl, F) analogues, introduction of the new chiral BP synthons **22a**, **22b**, **23a**, **23b**, **24a**, and **24b** required X-ray crystallographic analysis of the product nucleotides to assign the CHX configuration[28] of **35a** (Figure 6A) and **34b** (Figure 6B) (prepared from chiral BP synthons **24a** and **23b**, respectively).

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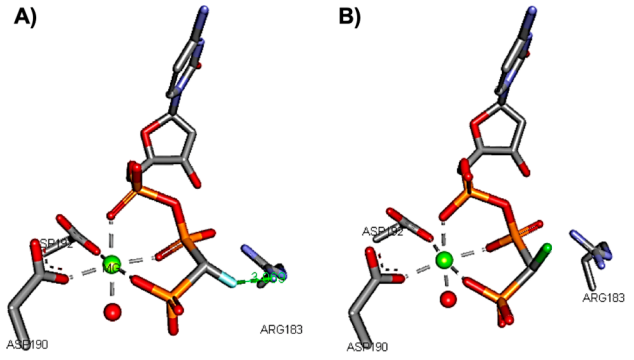

Figure 6. (A) X-ray crystallographic structure of (*R*)-*β*,*γ*-CHF-dCTP **35a** bound into the active site of a pol *β* ternary DNA complex (PDB entry 6BEL).[28] The Arg183, Asp190, and Asp192 side chains in the enzyme active site are shown, along with the nucleotide-binding magnesium and a water molecule. The interatomic distance between the F atom and Nη^2 of Arg183 is 3.07 Å. (B) Similar view of (*S*)-*β*,*γ*-CHCl-dCTP **34b** in the active site of pol *β* (PDB entry 6BEM).[28]

The ^{19}F and ^{31}P NMR spectra of the *β*,*γ*-CHF-dGTP stereoisomers **46a**, **46b** and the ^{31}P NMR spectra of the *β*,*γ*-CHCl-dGTP stereoisomers **45a**, **45b** suggested a possible correlation between their absolute CHX configurations and relative chemical shifts ($\Delta\delta_F$ 22.6 Hz and $\Delta\delta_{P\beta}$ 5.3 and 8.5 Hz, respectively).[35] This correlation finds further support from the NMR properties of the dTTP stereoisomers and is also observed in the dATP series (Table 1): $\delta_{P\beta}$ of the (*R*)-isomer is consistently observed slightly downfield (D) from $\delta_{P\beta}$ of the corresponding (*S*)-isomer. This was demonstrated for the dCTP analogues by a spiking experiment in which a small portion of (*S*)-*β*,*γ*-CHCl-dCTP **34b** was added to its (1:1) diastereomer mixture **34a/b** dissolved in D_2O (pH 10.0) (Figure 7**A**, **B**). The δ_F of the (*R*)-isomers resonated slightly upfield (U) from that of the (*S*)-isomers (Table 1), which was confirmed by adding a small portion of (*R*)-*β*,*γ*-CHF-dCTP **35a** or the (*S*)-*β*,*γ*-CHF-dATP diastereomer **12b** to the corresponding (1:1) mixtures **35a/b** (Figure 7C) and **12a/b** (Figure 7D), respectively. These assignments agree with the ^{19}F and ^{31}P $\Delta\delta$ predictions derived from our previous studies on *β*,*γ*-CHX-dGTP analogues (X = F, Cl).[35] Unlike $\delta_{P\beta}$, $\delta_{P\alpha}$ does not exhibit a consistent pattern when the new *β*,*γ*-CHX-dCTP analogue data are included (Table 1), possibly due to greater distance from the chiral CHX center.

Determination of the absolute configurations of the individual *β*,*γ*-CHX-dNTP (X = F, Cl) diastereomers by X-ray crystallographic analysis of their ternary complexes with pol *β*-DNA also defines the stereochemistry of the corresponding chiral bisphosphonate synthons precursors, revealing an interesting and useful correlation with their HPLC elution order (Table S1 and Figure 8). In examining stereochemical relationships among the several bisphosphonate synthons incorporating alternative chiral auxiliaries (Figure 4), we observe that a *"syn-syn"* configuration of the bulkiest aromatic group (phenyl in the case of the (*R*)-(+)-*α*-ethylbenzylamine and methyl (*R*)-(−)-phenylglycinate auxiliaries) relative to the X (F, Cl) methylene BP substituent always corresponds to the early eluted compound (fast isomer) during RP-HPLC separation, assuming the same HPLC column is used (Figure 8 and Figure 4C,D). This empirical rule can be rationalized on the basis of calculated dipole moments and correlated with logD values for the synthon stereoisomers (Table S1, entries 1−4, 7, 8, 17, 18, 21, 22, 25, and 26, and Figure S250) The same pattern is also seen for the P,C-dimorpholinamide derivatives (Figure 4A,B), in which the morpholine amide moiety is the bulkiest group. Opposite chirality at the CHX center generates a diastereomer pair ((*S*,*R*) and (*R*,*R*), Figure 4B; compare the (*R*,*S*) and (*S*,*S*) pair in Figure 4A) and reverses the HPLC elution order (Table S1, entries 1−4), as predicted by the polarity calculations. This empirical rule is followed by the *β*,*γ*-CHBr-dCTP and *β*,*γ*-CHCH$_3$-dATP diastereomers in which $\delta_{P\beta}$ for the (*R*)-isomer is again shifted downfield relative to that of the (*S*)-isomer (Figure 7E,F). The

Table 1. NMR Correlation with Absolute Configuration Assignment of Individual *β*,*γ*-CHX-dNTP Diastereomers (Dark Blue Denotes the Availability of a Crystal Structure)[28,35,36,43]

compd	*β*,*γ*-CHX-dNTP	^{31}P NMR P_α	^{31}P NMR P_β	^{19}F NMR	(*R*)- or (*S*)-	Absolute configuration assignment by	PDB entry	chiral auxiliary
11a	*β*,*γ*-CHCl-dTTP	U	D	N/A	(*R*)	crystal structure	6CTM	methyl (*R*)-(−) mandelate
11b	*β*,*γ*-CHCl-dTTP	D	U	N/A	(*S*)	crystal structure	6G2Q	methyl (*R*)-(−) mandelate
12a	*β*,*γ*-CHF-dATP	U	D	U	(*R*)	^{31}P NMR and ^{19}F NMR	N/A	methyl (*S*)-(+) mandelate
12b	*β*,*γ*-CHF-dATP	D	U	D	(*S*)	^{31}P NMR and ^{19}F NMR	N/A	methyl (*S*)-(+) mandelate
13a	*β*,*γ*-CHCl-dATP	U	D	N/A	(*R*)	^{31}P NMR	N/A	methyl (*R*)-(−) mandelate
13b	*β*,*γ*-CHCl-dATP	D	U	N/A	(*S*)	^{31}P NMR	N/A	methyl (*R*)-(−) mandelate
44a	*β*,*γ*-CHCH$_3$-dATP	D	D	N/A	(*R*)	^{31}P NMR	N/A	methyl (*R*)-(−) phenylglycinate
44b	*β*,*γ*-CHCH$_3$-dATP	U	U	N/A	(*S*)	^{31}P NMR	N/A	methyl (*R*)-(−) phenylglycinate
33a	*β*,*γ*-CHBr-dCTP	D	D	N/A	(*R*)	^{31}P NMR	N/A	(*R*)-(+)-*α*-ethylbenzylamine
33b	*β*,*γ*-CHBr-dCTP	U	U	N/A	(*S*)	^{31}P NMR	N/A	(*R*)-(+)-*α*-ethylbenzylamine
34a	*β*,*γ*-CHCl-dCTP	D	D	N/A	(*R*)	^{31}P NMR	N/A	(*R*)-(+)-*α*-ethylbenzylamine
34b	*β*,*γ*-CHCl-dCTP	U	U	N/A	(*S*)	crystal structure	6BEM	(*R*)-(+)-*α*-ethylbenzylamine
35a	*β*,*γ*-CHF-dCTP	D	D	U	(*R*)	crystal structure	6BEL	(*R*)-(+)-*α*-ethylbenzylamine
35b	*β*,*γ*-CHF-dCTP	U	U	D	(*S*)	^{31}P NMR and ^{19}F NMR	N/A	(*R*)-(+)-*α*-ethylbenzylamine
45a	*β*,*γ*-CHCl-dGTP	U	D	N/A	(*R*)	crystal structure	4DOB	methyl (*R*)-(−) mandelate
45b	*β*,*γ*-CHCl-dGTP	D	U	N/A	(*S*)	crystal structure	4DOC	methyl (*R*)-(−) mandelate
46a	*β*,*γ*-CHF-dGTP	U	D	U	(*R*)	crystal structure	4DO9	methyl (*R*)-(−) mandelate
46b	*β*,*γ*-CHF-dGTP	D	U	D	(*S*)	crystal structure	4DOA	methyl (*R*)-(−) mandelate

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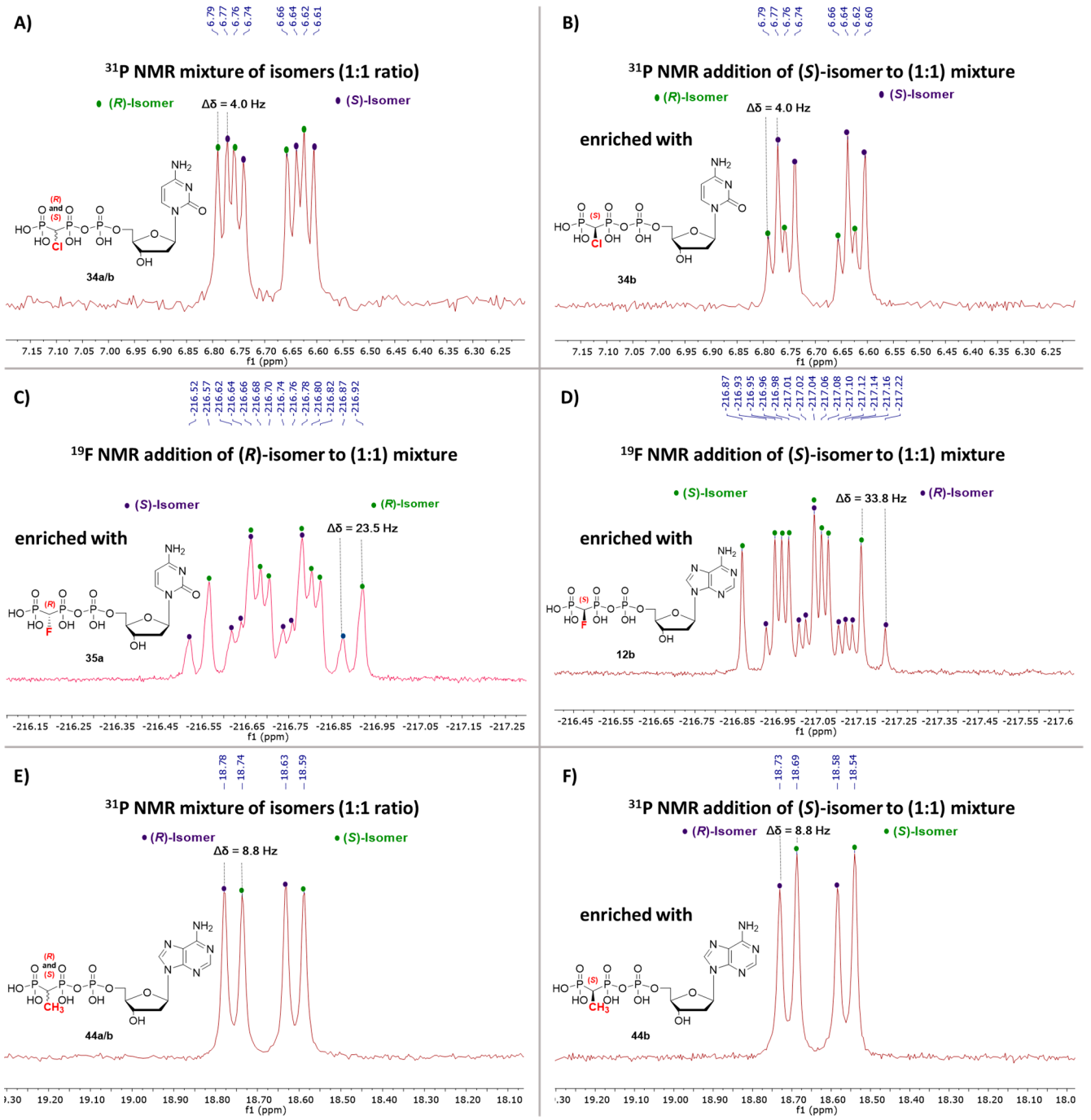

Figure 7. ^{31}P NMR (202 MHz, D_2O, pH 10.0): spectrum of (A) **34a/b**; (B) spiking experiment of **34a/b** with **34b** to identify isomer $\delta_{P\beta}$ values; (C) ^{19}F NMR (470 MHz, D_2O, pH 10.0): spiking experiment, **35a** added to **35a/b**; (D) ^{19}F NMR (564 MHz, D_2O, pH 10.0): spiking experiment, **12b** added to **12a/b**. ^{31}P NMR (202 MHz, D_2O, pH 10.0): (E) spectrum of **44a/b**; (F) spiking experiment, **44b** added to **44a/b**. (Green and purple color labeling corresponds respectively to the slow and fast eluting chiral synthons for the nucleotide analogues shown; see Figure 8. Note that reversal of the auxiliary chirality in the synthon is expected to reverse these "color" assignments, as demonstrated in the synthesis of **12a** and **12b**; compare Figure 4A and B).

absolute configuration assignments and derived empirical rules are summarized in Figure 8.

As mentioned above, the stereochemical logic of our approach requires that use of an auxiliary of opposite chirality in the bisphosphonate synthon should result in reversal of the HPLC elution order, with the more rapidly eluted synthon now generating a dNTP diastereomer with the opposite configuration at the β,γ-CHX. To verify this, we prepared the synthon **7c/d** from (*S*)-methyl mandelate, (**S**)-**3**, which separated into two peaks under the standard HPLC conditions as expected; however, the more rapidly eluted peak now yielded the (*R*)-β,γ-CHF-dATP diastereomer **12a**, and the more retained peak gave the opposite, (*S*)-β,γ-CHF-dATP diastereomer **12b**, which was confirmed by their ^{19}F and ^{31}P NMR spectra. Although we generally achieve baseline separation of the chiral bisphosphonate synthons using the HPLC methods we report here, under large scale-up conditions or when it is desired to obtain a particular β,γ-

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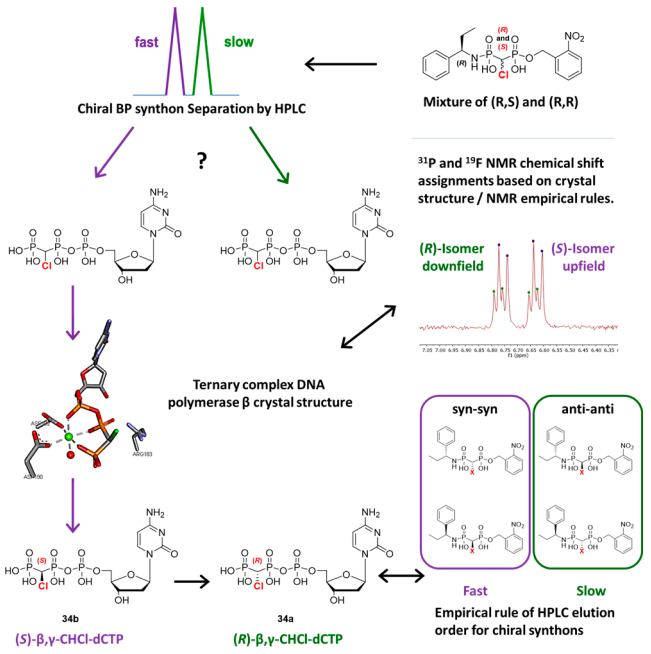

Figure 8. Example of absolute configuration assignments and observed correlations for the β,γ-CHCl-dCTP diastereomers and their chiral synthon precursors.

CHX-dNTP in very high stereochemical purity, the ability to select CHX chirality in the first-eluted synthon peak by changing the auxiliary chirality may be advantageous in minimizing cross-contamination.

The ultimate diastereopurity of the dNTP analogues will be limited by the enantiopurity of the chiral auxiliary used in their synthesis, assuming the absence of racemization. Given the limits of our analytical determinations and the quality of the available reagents, 98% or better diastereopurity can be obtained using our standard preparative conditions. In our original method, excessively high temperature (cf. preparation of the individual β,γ-CHCl-dATP diastereomers) may result in slightly lower diastereomeric purity, possibly due to <100% inversion of configuration during the Mitsunobu step.[58,59] This partial retention of configuration might be the reason for decreased diastereomeric purity in the synthesis of the individual β,γ-CFCl-dGTP diastereomers.[60] Avoidance of this step is thus a further advantage of our new routes.

Bisphosphonates as Leaving Groups. Acid dissociation constants are well-established parameters to quantify relative leaving group abilities in the construction of LFER plots.[61] For several bisphosphonic acids of interest in this work, acidity constants have not been published in the literature (CHCl, CBr_2, CFCl, $CFCH_3$, CHN_3, and $C(CH_3)N_3$), while the reported values for others vary significantly (up to a full pK_a unit), likely reflecting different measurement conditions (Table 2). In general, deviations in these literature values can be attributed, at least qualitatively, to effects resulting from variance in titration solution ionic strength, glass electrode size, temperature, and counterion.[62] An internally consistent set of pK_{a4} values for all the bisphosphonic acid components of our toolkit is essential for reliably rationalizing experimental kinetic results. We have therefore compiled experimentally determined $pK_{a(2-4)}$ values for the bisphosphonic acids and imidodiphosphoric acid (pNHp) used to complete the β,γ-CXY/NH toolkit, including all the pK_{a4} values used in our published LFER studies, obtained under the same conditions.[27,32]

The pK_a values are presented in Table 2, and experimental data are listed in the Supporting Information. All acidity constants in our laboratory were determined at constant temperature (25 °C) by potentiometric titration under Ar of the free acid form of the bisphosphonate in CO_2−free H_2O containing 0.1 M KCl with 0.1 M KOH as the titrant using an automated Schott Instruments Titrator Basic. The data was fitted to a calculated titration curve using Hyperquad2006 or 2008[63] to obtain acidity constants using standard equations.

CONCLUSION

In conclusion, with the goal of completing the β,γ-CXY-dNTP stereochemical probes "toolkit" we have devised an alternative synthetic strategy featuring novel chiral BP synthons equipped with an auxiliary chiral amine ((*R*)-(+)-α-ethylbenzylamine or methyl (*R*)-(−)-phenylglycinate) and a photochemically cleavable *o*-nitrobenzyl group. Using this new approach, we have obtained previously inaccessible individual β,γ-CHX-dCTP (X = F, Cl, and Br) diastereomers, and the versatility of the method was demonstrated by synthesis of the novel individual β,γ-$CHCH_3$-dATP diastereomers. Individual β,γ-CHX diastereomers of dATP (X = F, Cl) and dTTP (X = Cl) were also obtained via P,C-dimorpholinamide bisphosphonate derivatives. The absolute configurations of the diastereomers, determined by X-ray crystallographic analysis of their ternary complexes with pol β−DNA, defines the stereochemistry of the corresponding chiral bisphosphonate synthon precursors. We demonstrate that the (*R*)- and (*S*)-β,γ-CHX-dNTPs can be analytically distinguished by their P_β ^{31}P NMR and ^{19}F NMR δ values. Finally, a consistent set of pK_{a2-4} values for 13 CXY-bisphosphonic acids and imidodiphosphoric acid determined by potentiometric titration is compiled. These data are essential for any LFER analysis of leaving group effects on a chemical transition state in polymerase-catalyzed dNTP analogue turnover, which in turn is fundamental to understanding the catalytic mechanisms. The toolkit should also find wider application to access a broad range of chiral BP derivatives for exploring phosphate and pyrophosphate biochemistry in biological processes.

EXPERIMENTAL SECTION

Materials and Methods. Adenosine-, thymidine-, and cytidine-5′-monophosphoric acids were purchased from Chem-Impex International. All phosphonic esters and bisphosphonic acids were prepared according to the literature.[35,37,48−50] Purification of tetraalkyl bis(phosphonate) esters was preferably performed using an ISCO CombiFlashRf+ Lumen flash chromatography system equipped with an ELSD detector. All other reagents were purchased from Sigma-Aldrich, Fluka, or Alfa Aesar (reagent grade) and used as received. Synthesis of the individual diastereomers of β,γ-CHF and β,γ-CHCl-dATP and also β,γ-CHCl-dTTP was performed using our published method.[35] Synthesis of individual diastereomers of β,γ-CHBr-, -CHCl-, and -CHF-dCTP and β,γ-$CHCH_3$-dATP was accomplished using the new methods reported herein. ^{1}H, ^{13}C, ^{31}P, and ^{19}F NMR spectra were obtained on a Varian 400-MR, VNMRS-500, or VNMRS-600 spectrometer. All ^{1}H and ^{13}C peak assignments were verified by COSY and HSQCAD. ^{31}P NMR spectra were proton-decoupled unless stated otherwise. Multiplicities are quoted as singlet (s), doublet (d), triplet (t), unresolved multiplet (m), doublet of doublets (dd), doublet of doublet of doublets (ddd), doublet of triplets (dt), triplet of doublets (td), and broad (b). All chemical shifts (δ) are reported in parts per million (ppm) relative to residual CD_2HOD in CD_3OD (δ 3.34, ^{1}H NMR), $CHCl_3$ in $CDCl_3$ (δ 7.26,

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Table 2. Acidity Constants for Bisphosphonic Acids and Imidophosphoric Acid

		our work		
CXY/NH	lit. pK_{a4} values	pK_{a4}	pK_{a3}	pK_{a2}
CF_2	7.63,[64] *I*: 0.1 M NaCl; *t*: 25°	7.77 ± 0.04	5.63 ± 0.04	1.69 ± 0.2
	8.00,[65] *I*: 0.0 M Na^+; *t*: 20°			
	8.14,[66] *I*: 0.15 M Me_4NCl; *t*: 25°			
	8.16,[67] *I*: 0.1 M Me_4NCl; *t*: 25°			
CFCl		8.36 ± 0.03	5.58 ± 0.02	1.58 ± 0.1
CCl_2	8.30,[67] *I*: 0.1 M Me_4NCl; *t*: 25°	8.88 ± 0.04	5.79 ± 0.02	2.00 ± 0.07
	8.84[b],[68] *I*: 0.1 M KCl; *t*: 25°			
	9.5[a],[69] *I*: 0.1 M Me_4NCl; *t*: 25°			
	9.50,[70] *I*: 0.1 M Me_4NCl; *t*: 25°			
	9.72,[71] *I*: 0.1 M Me_4NNO_3; *t*: 25°			
	9.78,[65] *I*: 0.0 M Na^+; *t*: 20°			
CHF	9.35,[65] *I*: 0.0 M Na^+; *t*: 20°	9.01 ± 0.03	6.15 ± 0.02	1.30 ± 0.4
	9.44,[66] *I*: 0.15 M Me_4NCl; *t*: 25°			
CBr_2		9.27 ± 0.003	5.87 ± 0.006	1.83 ± 0.03
CHN_3		9.39 ± 0.008	6.26 ± 0.02	2.08 ± 0.05
CHCl		9.58 ± 0.06	6.32 ± 0.02	1.19 ± 0.4
CHBr	10.00,[72] *I*: 0.032 M Me_4NCl; *t*: 25°	9.91 ± 0.009	6.16 ± 0.03	2.33 ± 0.1
$CFCH_3$		10.20 ± 0.01	6.21 ± 0.003	1.86 ± 0.02
$C(CH_3)N_3$		10.50 ± 0.05	6.40 ± 0.01	2.33 ± 0.02
$CHCH_3$		11.59 ± 0.02	7.05 ± 0.04	2.73 ± 0.05
$C(CH_3)_2$		12.24 ± 0.05	7.67 ± 0.01	2.88 ± 0.03
CH_2	9.89,[73] *I*: 0.10 M NaCl; *t*: 25°	10.52 ± 0.007	6.92 ± 0.001	2.75 ± 0.05
	10.00,[74] *I*: 0.2 M KCl; *t*: 25°			
	10.42[c],[75] *I*: 0.10 M KCl; *t*: 25°			
	10.54,[76] *I*: 0.5 M Me_4NCl; *t*: 25°			
	10.57,[70] *I*: 0.10 M Me_4NCl; *t*: 25°			
	10.75,[71] *I*: 0.10 M Me_4NNO_3; *t*: 25°			
	10.75,[77] *I*: 0.10 M KNO_3; *t*: 25°			
	10.96,[65] *I*: 0.0 M Na^+; *t*: 20°			
NH	10.22,[78] *I*: 0.10 M Me_4NBr; *t*: 25°	9.70 ± 0.022	7.11 ± 0.04	2.81 ± 0.05
	9.72,[78] *I*: 0.2 M Me_4NBr; *t*: 25°			
	9.77,[78] *I*: 0.3 M Me_4NBr; *t*: 25°			
	10.36,[78] *I*: 1.0 M Me_4NBr; *t*: 25°			
	9.79,[79] *I*: 0.1 M Me_4NBr; *t*: 37°			
	9.52,[79] *I*: 0.3 M Me_4NBr; *t*: 37°			
	9.41,[79] *I*: 0.1 M Me_4NBr; *t*: 50°			
	9.32,[79] *I*: 0.3 M Me_4NBr; *t*: 50°			

[a]IUPAC provisional value. [b]pK_{a3}: 5.82 and pK_{a2}: 2.3. [c]pK_{a3}: 7.33 and pK_{a2}: 2.75.

^{1}H NMR), HDO in D_2O (δ 4.80, ^{1}H NMR), external 85% H_3PO_4 (δ 0.00, ^{31}P NMR) or external C_6F_6 (δ −164.9, ^{19}F NMR). pH of the NMR samples was adjusted to 10 (unless stated otherwise) or higher (using sodium carbonate) which is critical to obtain high resolution ^{31}P NMR spectra.[57] The pH meter measurements were calibrated at three different pH values (4, 7, and 10) using standard buffers. NMR spectra processing was performed with MestReNova 9.0.0 or 11.0.2. Preparative HPLC was performed using a Varian ProStar or Shimadzu Prominence instrument equipped with a Shimadzu SPD-20A UV detector (0.5 mm path length) with detection at 280 nm for *o*-nitrobenzyl derivatives and at 260 nm for methyl mandelate derivatives and all dNTP analogues. Strong Anion Exchange (SAX) HPLC was performed on a Macherey Nagel 21.4 mm × 250 mm SP15/25 Nucleogel column. RP HPLC was performed on a Phenomenex Luna 5 μm C18(2) 100A 21.2 mm × 250 mm column. Time-of-flight high resolution mass spectrometry (TOF-HRMS) was performed on a Water Synapt G2-Si ESI spectrometer (performed at the School of Chemical Sciences Mass Spectrometry Laboratory (MSL) at the University of Illinois) and low-resolution mass spectrometry on a Finnigan LCQ Deca XP Max mass spectrometer equipped with an ESI source, both in the negative ion mode. MS m/z values were calculated using ChemDraw 15.0.0.106 or iMass 1.3. Compound IUPAC names were assigned using MarvinSketch 16.12.12. The molar yields of the final products were estimated by UV absorbance referenced to the extinction coefficient of dCTP at pH 7.0 (phosphate buffer) at 271 nm (ε cytidine = 8860), of dATP at pH 7.0 (phosphate buffer) at 259 nm (ε adenosine = 15080) or of dTTP at pH 7.0 (phosphate buffer) at 267 nm (ε thymidine = 9490).[80] The "slow" and "fast" chiral synthon HPLC peak descriptors reflect elution order on the RP-C18 column. Compounds numbering #a (or c) and #b (or d) correspond respectively to synthetic pathway of their final (*R*)- and (*S*)-β,γ-CHX-dNTP analogues.

Synthesis of β,γ-CXY-dATP Analogues. General Method 1 (Synthesis of β,γ-CXY-dATPs 12a/b, 13a/b, 44a/b, 47a/b, and 45−49, See Figure 1). To a solution of the appropriate CXY bisphosphonic acid (3−4 equiv) in a mixture of EtOH/H_2O (1:1) ~[0.1 M] was added $(Bu_3)N$ to adjust the pH to 3−5, and the mixture was stirred for 15 min. The solvent was removed under reduced pressure, and the residue coevaporated (3×) with anhydrous DMF. The 5′-phosphoromorpholidate[52] of the dAMP (1.0 equiv, 0.31 mmol) in anhydrous DMSO (0.1 M) was added, and the solution was stirred at rt for 2 d while monitored by ^{31}P NMR. When the reaction was complete, the crude product was purified by dual-pass preparative HPLC: first on a preparative SAX HPLC column

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(8.0 mL/min, 259 nm) in gradient mode (A/H_2O and B/0.5 M triethylammonium bicarbonate pH 8.0 buffer: 0−10 min B/0%−40%, 10−15 min B/40%, 15−25 min b/40%−100%) and then on a preparative RP-C18 column (8.0 mL/min, 259 nm) in isocratic mode (6.5% acetonitrile in 0.1 M triethylammonium bicarbonate, pH 8.0). The final product yield was ∼30%, and its purity (analytical SAX HPLC and RP-C18) was ≥99%.

({[({[(2R,5R)-5-(6-Amino-9H-purin-9-yl)-3-hydroxyoxolan-2-yl]methoxy}(hydroxy)phosphoryl)oxy](hydroxy)phosphoryl}methyl)phosphonic Acid, β,γ-CH_2-dATP ***45***. According to general method 1, (phosphonomethyl)phosphonic acid (176 mg, 1.0 mmol) in 10 mL of EtOH/H_2O (1:1) was reacted with Bu_3N (450 μL, 1.5 mmol) and 2′-deoxyadenosine 5′-phosphoromorpholidate (120.0 mg, 0.3 mmol) in 3 mL of anhydrous DMSO for 2 d. ^{31}P NMR (162 MHz, D_2O, pH 11.5) δ 15.25 (dd, J = 27.6, 7.5 Hz, 1P), 13.88 (d, J = 7.3 Hz, 1P), −8.45 (d, J = 27.7 Hz, 1P). ^1H NMR (400 MHz, D_2O): δ 8.36 (s, 1H), 8.10 (s, 1H), 6.43−6.27 (m, 1H), 4.73−4.54 (m, 1H), 4.15 (s, 1H), 4.03 (dt, J = 15.1, 4.8 Hz, 2H), 2.76−2.60 (m, 1H), 2.46 (m, 1H), 2.19 (t, J = 20.3 Hz, 2H). HRMS (ESI-TOF) m/z: $[M - H]^-$ calcd for $C_{11}H_{17}N_5O_{11}P_3^-$ 488.0137; found 488.0138. Crystal structure available at PDB entry 6CR5.[28]

({[({[(2R,5R)-5-(6-Amino-9H-purin-9-yl)-3-hydroxyoxolan-2-yl]methoxy}(hydroxy)phosphoryl)oxy](hydroxy)phosphoryl}(chloro)methyl)phosphonic Acid, β,γ-CHCl-dATP ***13a/b*** *Mixture of Diastereomers*. According to general method 1, [chloro(phosphono)methyl]phosphonic acid **15** (210 mg, 1.0 mmol) in 10 mL of EtOH/H_2O (1:1) was reacted with Bu_3N (450 μL, 1.5 mmol) and 2′-deoxyadenosine 5′-phosphoromorpholidate (120.0 mg, 0.3 mmol) in 3 mL of anhydrous DMSO for 2 d. ^{31}P NMR (202 MHz, D_2O, pH 11.5): δ 9.61 (d, J = 6.2 Hz, 1P), 2.89 (dd, J = 26.6, 6.3 Hz, 1P), avg −11.26 (d, J = 26.9 Hz, 1P), (Δδ = 0.18 ppm). ^1H NMR (500 MHz, D_2O): δ 8.34 (s, 1H), avg 8.07 (Δδ = 0.006 ppm, 1H), 6.35 (t, J = 6.8 Hz, 1H), 4.67 (m, 1H), 4.16 (b, 1H), 4.05 (m, 2H), 3.89 (dd, J = 16.3 Hz, 1H), 2.70 (m, 1H), 2.47 (m, 1H). ^{13}C NMR (126 MHz, D_2O): δ 155.10 (C6), 152.43 (C8), 148.38 (C4), 139.74 (C2), 118.22 (C5), 85.69 (C4′), 83.43 (C1′), 71.05 (C3′), 65.25 (C5′), 48.21 (β,γ-CHCl, dd, J = 137.1, 126.4 Hz), 38.92 (C2′). HRMS (ESI-TOF) m/z: $[M - H]^-$ calcd for $C_{11}H_{16}ClN_5O_{11}P_3^-$ 521.9748; found 521.9752. Crystal structure available at PDB entry 6CR8.[28]

({[({[(2R,5R)-5-(6-Amino-9H-purin-9-yl)-3-hydroxyoxolan-2-yl]methoxy}(hydroxy)phosphoryl)oxy](hydroxy)phosphoryl}(fluoro)methyl)phosphonic Acid, β,γ-CHF-dATP ***12a/b*** *Mixture of Diastereomers*. According to general method 1, [fluoro(phosphono)methyl]phosphonic acid **16** (194 mg, 1.0 mmol) in 10 mL of EtOH/H_2O (1:1) was reacted with Bu_3N (450 μL, 1.5 mmol) and 2′-deoxyadenosine 5′-phosphoromorpholidate (120.0 mg, 0.3 mmol) in 3 mL of anhydrous DMSO for 2 d. ^{19}F NMR (470 MHz, D_2O, pH 10) avg δ −219.47 (2 × ddd/overlap, J = 61, 45, 15 Hz). ^{31}P NMR (202 MHz, D_2O, pH 10): δ 8.11 (dd, J = 61, 14 Hz, 1P), 0.97 (ddd, J = 62, 28, 14 Hz, 1P), −11.21 (d, J = 28 Hz). ^1H NMR (500 MHz, D_2O): δ 8.36 (s, 1H), 8.11 (s, 1H), 6.52−6.23 (m, 1H), 4.81 (dt, J = 45.7, 12.9 Hz, 1H), 4.67 (m, 1H), 4.25−4.12 (m, 1H), 4.14−3.95 (m, 2H), 2.70 (m, 1H), 2.47 (m, 1H). ^{13}C NMR (126 MHz, D_2O): δ 155.50 (C6), 152.63 (C8), 148.66 (C4), 139.95 (C2), 118.48 (C5), 89.52 (β,γ-CHF, ddd, J = 178.7, 149.3, 134.9 Hz), 85.80 (C4′), 83.47 (C1′), 70.94 (C3′), 65.04 (C5′), 38.94 (C2′). HRMS (ESI-TOF) m/z: $[M - H]^-$ calcd for $C_{11}H_{16}FN_5O_{11}P_3^-$ 506.0043; found 506.0056. Crystal structure available at PDB entry 6CR7.[28]

({[({[(2R,5R)-5-(6-Amino-9H-purin-9-yl)-3-hydroxyoxolan-2-yl]methoxy}(hydroxy)phosphoryl)oxy](hydroxy)phosphoryl}difluoromethyl)phosphonic Acid, β,γ-CF_2-dATP ***46***. According to general method 1, [difluoro(phosphono)methyl]phosphonic acid (212 mg, 1.0 mmol) in 10 mL of EtOH/H_2O (1:1) was reacted with Bu_3N (450 μL, 1.5 mmol) and 2′-deoxyadenosine 5′-phosphoromorpholidate (120.0 mg, 0.3 mmol) in 3 mL of anhydrous DMSO for 2 d. ^{19}F NMR (564 MHz, D_2O, pH 10): δ −119.60 (app. t, J = 84 Hz). ^{31}P NMR (243 MHz, D_2O, pH 10): δ 2.91 (td, J = 80.0, 58.7 Hz, 1P), −4.26 to −6.53 (m, 1P), −11.20 (d, J = 31 Hz, 1P). ^1H NMR (600 MHz, D_2O): δ 8.34 (s, 1H), 8.09 (s, 1H), 6.36 (dd, J = 7.5, 6.2 Hz, 1H), 4.65 (m, 1H), 4.15 (m, 1H), 4.08 (m, 1H), 4.00 (m, 1H), 2.68 (m, 1H), 2.44 (m, 1H). HRMS (ESI-TOF) m/z: $[M - H]^-$ calcd for $C_{11}H_{15}F_2N_5O_{11}P_3^-$ 523.9949; found 523.9949. Crystal structure available at PDB entry 6CRA.[28]

({[({[(2R,5R)-5-(6-Amino-9H-purin-9-yl)-3-hydroxyoxolan-2-yl]methoxy}(hydroxy)phosphoryl)oxy](hydroxy)phosphoryl}(chloro)fluoromethyl)phosphonic Acid, β,γ-CFCl-dATP ***47a/b*** *mixture of diastereomers*. According to general method 1, [chloro(fluoro)phosphonomethyl]phosphonic acid (228 mg, 1.0 mmol) in 10 mL of EtOH/H_2O (1:1) was reacted with Bu_3N (450 μL, 1.5 mmol) and 2′-deoxyadenosine 5′-phosphoromorpholidate (120.0 mg, 0.3 mmol) in 3 mL of anhydrous DMSO for 2 d. ^{19}F NMR (376 MHz, D_2O, pH 10) avg δ −136.98 (2 × dd, J = 78, 64 Hz). ^{31}P NMR (162 MHz, D_2O, pH 10): δ 5.95 (dd, J = 64, 33 Hz, 1P), −0.36 (Δδ = 0.02 ppm, 2 × ddd, J = 79, 34, 31 Hz, 1P), −10.73 (d, J = 31 Hz, 1P). ^1H NMR (500 MHz, D_2O) avg δ 8.37 (Δδ = 0.01 ppm, 1H), 8.11 (Δδ = 0.01 ppm, 1H), 6.38 (app. t, J = 6.7 Hz, 1H), 4.68 (m, 1H), 4.26−3.93 (m, 3H), 2.70 (m, 1H), 2.46 (m, 1H). ^{13}C NMR (126 MHz, D_2O): δ 155.28 (C6), 152.48 (C8), 148.49 (C4), 139.86 (C2), 118.30 (C5), 109.34−103.86 (β,γ-CFCl, m), 85.75 (C4″), 83.41 (C1′), 70.90 (C3′), 65.25 (C5′), 38.90 (C2′). HRMS (ESI-TOF) m/z: $[M - H]^-$ calcd for $C_{11}H_{15}FClN_5O_{11}P_3^-$ 539.9653; found 539.9664. Crystal structure available at PDB entry 6CR9.[28]

({[({[(2R,5R)-5-(6-Amino-9H-purin-9-yl)-3-hydroxyoxolan-2-yl]methoxy}(hydroxy)phosphoryl)oxy](hydroxy)phosphoryl}dichloromethyl)phosphonic Acid, β,γ-CCl_2-dATP ***48***. According to general method 1, [dichloro(phosphono)methyl]phosphonic acid (245 mg, 1.0 mmol) in 10 mL of EtOH/H_2O (1:1) was reacted with Bu_3N (450 μL, 1.5 mmol) and 2′-deoxyadenosine 5′-phosphoromorpholidate (120.0 mg, 0.3 mmol) in 3 mL of anhydrous DMSO for 2 d. ^{31}P NMR (162 MHz, D_2O, pH 10): δ 4.70 (d, J = 19 Hz, 1P), −3.62 (dd, J = 29.5, 19 Hz, 1P), −13.58 (d, J = 29.5 Hz, 1P). ^1H NMR (400 MHz, D_2O): δ 8.36 (s, 1H), 8.11 (s, 1H), 6.38 (t, J = 7.0 Hz, 1H), 4.81−4.57 (m, 1H), 4.23−3.95 (m, 3H), 2.71 (m, 1H), 2.46 (m, 1H). HRMS (ESI-TOF) m/z: $[M - H]^-$ calcd for $C_{11}H_{15}Cl_2N_5O_{11}P_3^-$ 555.9358; found 555.9372. Crystal structure available at PDB entry 6CRB.[28]

({[({[(2R,5R)-5-(6-Amino-9H-purin-9-yl)-3-hydroxyoxolan-2-yl]methoxy}(hydroxy)phosphoryl)oxy](hydroxy)phosphoryl}dibromomethyl)phosphonic Acid, β,γ-CBr_2-dATP ***49***. According to general method 1, [dibromo(phosphono)methyl]phosphonic acid (334 mg, 1.0 mmol) in 10 mL of EtOH/H_2O (1:1) was reacted with Bu_3N (450 μL, 1.5 mmol) and 2′-deoxyadenosine 5′-phosphoromorpholidate (120.0 mg, 0.3 mmol) in 3 mL of anhydrous DMSO for 2 d. ^{31}P NMR (202 MHz, D_2O, pH 10): δ 7.81 (d, J = 14.5 Hz, 1P), 2.11 (dd, J = 30, 14.5 Hz, 1P), −10.78 (d, J = 30 Hz, 1P). ^1H NMR (500 MHz, D_2O): δ 8.37 (s, 1H), 8.1 (s, 1H), 6.54−6.23 (m, 1H), 4.81−4.67 (m, 1H), 4.27−3.95 (m, 3H), 2.71 (m, 1H), 2.47 (m, 1H). ^{13}C NMR (126 MHz, D_2O): δ 155.27 (C6), 152.48 (C8), 148.45 (C4), 139.86 (C2), 118.28 (C5), 85.78 (C4′), 83.39 (C1′), 70.86 (C3′), 65.29 (C5′), 58.50 (β,γ-CBr_2, dd, J = 133.8, 115.1 Hz), 38.90 (C2′). HRMS (ESI-TOF) m/z: $[M - H]^-$ calcd for $C_{11}H_{15}Br_2N_5O_{11}P_3^-$ 643.8348; found 643.8359. Crystal structure available at PDB entry 6CR3.[28]

(1-{[({[(2R,5R)-5-(6-Amino-9H-purin-9-yl)-3-hydroxyoxolan-2-yl]methoxy}(hydroxy)phosphoryl)oxy](hydroxy)phosphoryl}ethyl)phosphonic Acid, β,γ-CH(CH_3)-dATP ***44a/b*** *Mixture of Diastereomers*. According to general method 1, (1-phosphonoethyl)phosphonic acid **36** (190 mg, 1.0 mmol) in 10 mL of EtOH/H_2O (1:1) was reacted with Bu_3N (450 μL, 1.5 mmol) and 2′-deoxyadenosine 5′-phosphoromorpholidate (120.0 mg, 0.3 mmol) in 3 mL of anhydrous DMSO for 2 d. ^{31}P NMR (162 MHz, D_2O, pH 10): δ 19.75 (b, 1P), 14.09 (d, J = 29 Hz, 1P), avg −11.11 (Δδ = 0.04 ppm, J = 29 Hz, 1P). ^1H NMR (500 MHz, D_2O): δ 8.34 (s, 1H), avg 8.07 (Δδ = 0.005 ppm, 1H), 6.34 (t, J = 7 Hz, 1H), 4.68 (s, 1H), 4.15 (m, 1H), 4.03 (m, 2H), 2.69 (m, 1H), 2.46 (m, 1H), 2.04 (tq, J = 23, 7.5 Hz, 1H), 1.24 (dt, J = 18, 7.5 Hz, 3H). ^{13}C NMR (151 MHz, D_2O): δ 153.81 (C6), 150.51 (C8), 148.26 (C4), 140.46 (C2), 118.25 (C5), 85.77 (C4′), 83.70 (C1′), 70.93 (C3′), 65.14 (C5′), 39.12 (C2′), 33.67 (CH in β,γ-CH(CH_3), dd, J = 129.3, 121.2 Hz), 10.50−1043 (m, CH_3 in β,γ-CH(CH_3)). HRMS (ESI-TOF) m/z: $[M - H]^-$ calcd for $C_{12}H_{19}N_5O_{11}P_3^-$ 502.0294; found 502.0299. Crystal structure available at PDB entry 6CR6.[28]

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Synthesis of Individual Diastereomers of β,γ-CHX-dCTP (X = Br, Cl, F) Analogues 33a, 33b, 34a, 34b, 35a, and 35b. Introduction of the 2-Nitrobenzyl Group 17. *(Bromo(hydroxy-((2-nitrobenzyl)oxy)phosphoryl)methyl)phosphonic Acid **18a/b**.* A solution of [bromo(phosphono)methyl]phosphonic acid **14** (510 mg, 2.0 mmol), 2-nitrobenzyl bromide **17** (215 mg, 1.0 mmol), and DIEA (1.53 mL, 8.8 mmol) in anhydrous DMF (16 mL) was heated at 80 °C for 90 min. The reaction was monitored by ^{31}P NMR. After 2.5 h, solvent was removed under reduced pressure, the residue was dissolved in ethyl acetate, and the target compound was extracted with water. The solvent was removed under reduced pressure and the crude mixture was purified by preparative RP-C18 HPLC, isocratic mode, using 18.5% acetonitrile in 0.1 M triethylammonium bicarbonate, pH 7.5, at a flow rate of 8.0 mL/min and UV detection (280 nm); the title compound eluted at 8.2 min. After removal of solvents under reduced pressure and coevaporation with methanol the desired product was obtained as bis(triethylammonium) salts (426 mg, 36%). Colorless oil. ^{31}P NMR (162 MHz, D_2O, pH 10): δ 14.94 (b, 1P), 7.5 (b, 1P). ^{1}H NMR (500 MHz, D_2O): δ 8.06 (dd, *J* = 8.2, 1.2 Hz, 1H), 7.87 (d, *J* = 7.9 Hz, 1H), 7.69 (t, *J* = 7.6, 1H), 7.44 (t, *J* = 7.9, 1H), 5.38−5.32 (m, 2H), 3.74 (t, *J* = 15.1 Hz, 1H). MS (ESI) *m/z*: $[M - H]^-$ calcd for $C_8H_9BrNO_8P_2^-$ 387.89; found 388.0.

*(Chloro(hydroxy((2-nitrobenzyl)oxy)phosphoryl)methyl)-phosphonic Acid **19a/b**.* A solution of [chloro(phosphono)methyl]-phosphonic acid **15** (189 mg, 0.9 mmol), 2-nitrobenzyl bromide **17** (302 mg, 1.4 mmol), and DIEA (1.38 mL, 7.9 mmol) in anhydrous DMF (15 mL) was heated at 80 °C for 90 min. The reaction was monitored by ^{31}P NMR. The crude mixture was purified by preparative RP-C18 HPLC, isocratic mode, using 18.5% acetonitrile in 0.1 M triethylammonium carbonate, pH 8.5, at a flow rate of 8.0 mL/min and UV detection (280 nm); the title compound **19a/b** eluted at 8.5 min. After removal of solvents under reduced pressure and coevaporation with methanol, the desired product was obtained as bis(triethylammonium) salts (158 mg, 32%). ^{31}P NMR (202 MHz, D_2O, pH 10): δ 13.14 (d, *J* = 4 Hz, 1P), 9.51 (d, *J* = 4 Hz, 1P). ^{1}H NMR (500 MHz, D_2O): δ 8.03 (dd, *J* = 8, 1 Hz, 1H), 7.85 (d, *J* = 8 Hz, 1H), 7.67 (td, *J* = 8, 1 Hz, 1H), 7.48−7.35 (m, 1H), 5.43−5.21 (m, 2H), 3.78 (t, *J* = 15.5 Hz, 1H).

*(Fluoro(hydroxy((2-nitrobenzyl)oxy)phosphoryl)methyl)-phosphonic Acid **20a/b**.* To a prewarmed solution of [fluoro-(phosphono)methyl]phosphonic acid **16** (1.22 g, 6.3 mmol) in anhydrous DMF (315 mL) at 125 °C was added DIEA (1.2 mL, 6.9 mmol) dropwise, and the mixture stirred for 15 min at 125 °C. A solution of 2-nitrobenzyl bromide **17** (1.49 g, 6.9 mmol) in DMF (35 mL) was then slowly added (over 15 min) through the condenser, and the reaction mixture was kept at 125 °C for 24 h. Reaction progress was monitored by ^{31}P NMR and MS. After completion, the resulting mixture was diluted with ethyl acetate at rt and evaporated to dryness. The residue was purified by preparative RP-C18 HPLC, isocratic mode, using 15% acetonitrile in 0.1 M triethylammonium bicarbonate, pH 7.5, at a flow rate of 8.0 mL/min and UV detection (280 nm). The desired fraction was evaporated to dryness and the desired product was obtained as bis(triethylammonium) salts (2.3 g, 69%). ^{19}F NMR (376 MHz, D_2O, pH 10): δ −217.91 (ddd, *J* = 62, 55, 45 Hz). ^{31}P NMR (162 MHz, D_2O, pH 10): δ 14.19 (dd, *J* = 62, 11 Hz, 1P), 7.30 (dd, *J* = 55, 11 Hz, 1P). ^{1}H NMR (500 MHz, D_2O): δ 8.18 (dd, *J* = 8, 1 Hz, 1H), 8.00 (d, *J* = 8 Hz, 1H), 7.87−7.78 (m, 1H), 7.56 (t, *J* = 8 Hz, 1H), 5.45 (d, *J* = 8 Hz, 2H), 4.95−4.85 (m, 1H). MS (ESI) *m/z*: $[M - H]^-$ calcd for $C_8H_9FNO_8P_2^-$ 327.97; found 328.1.

Preparation of the Separable Chiral Synthons. General Method 2. To a solution of the corresponding bisphosphonate derivative **18a/b**, **19a/b**, or **20a/b** (1.0 equiv) in anhydrous DMSO or DMF at rt under nitrogen were added the auxiliary chiral amine **21** (3−10 equiv), 2, 2′-dithiodipyridine (1−3 equiv), and triphenylphosphine (1−3 equiv). The reaction mixture stirred at rt for 3 to 16 h and monitored by ^{31}P NMR and MS. The crude material was purified by preparative RP-C18 HPLC as indicated for each case.

*[(S)-Bromo[hydroxy({[(1R)-1-phenylpropyl]amino})phosphoryl]-methyl][(2-nitrophenyl)methoxy]phosphinic Acid **22a** and Its (R) Isomer **22b**.* Using general method 2, the α-bromo methylenebis-(phosphonate) derivative **18a/b** as its bis(triethylammonium) salt form (0.7 mmol) in DMSO (1.0 mL) was reacted with (*R*)-1-phenylpropan-1-amine **21** (590 μL, 4.1 mmol), 2,2′-dithiodipyridine (308 mg, 1.4 mmol), and triphenylphosphine (367 mg, 1.4 mmol) for 16 h at rt. Purification was performed on the SAX column, isocratic mode, using 10% acetonitrile in 0.5 M triethylammonium bicarbonate, pH 7.9, at a flow rate of 8.0 mL/min and UV detection (280 nm); the title compounds **22a/b** eluted at 8.5 min. After removal of solvent under reduced pressure, separation of the diastereomers was performed using preparative RP-C18 HPLC, isocratic mode, using 32% acetonitrile in 0.1 M triethylammonium carbonate buffer, pH 8.5, at a flow rate of 8.0 mL/min and UV detection (280 nm). Total (isomer ratio 1:1) yield 29% (by NMR).

The slow diastereomer, (*S*)-CHBr-(*R*)-auxiliary **22a** was eluted at 11.7 min: ^{31}P NMR (202 MHz, CD_3OD): δ 11.53 (b, 1P), 10.71 (b, 1P). ^{1}H NMR (500 MHz,CD_3OD): δ 8.19 (m, 1H), 7.81 (m, 1H), 7.60 (m, 1H), 7.62−7.48 (m, 3H), 7.37 (t, *J* = 7.5 Hz, 2H), 7.24 (t, *J* = 7.4 Hz, 1H), 5.59−5.41 (m, 2H), 4.42 (q, *J* = 7.1 Hz, 1H), 3.44 (s, 1H), 2.06−1.99 (m, 1H), 1.88−1.78 (m, 1H), 0.94 (q, *J* = 7.1 Hz, 3H). MS (ESI) *m/z*: $[M - H]^-$ calcd for $C_{17}H_{20}BrN_2O_7P_2^-$ 504.99; found 505.2.

The fast diastereomer, (*R*)-CHBr-(*R*)-auxiliary **22b** was eluted at 10.4 min: ^{31}P NMR (202 MHz, CD_3OD): δ 11.54 (b, 1P), 10.29 (b, 1P). ^{1}H NMR (500 MHz, CD_3OD): δ 8.30 (m, 1H), 7.91 (m, 1H), 7.74−7.66 (m, 1H), 7.63−7.52 (m, 3H), 7.44 (d, *J* = 8.5 Hz, 1H), 7.34 (d, *J* = 8.4 Hz, 1H), 5.74−5.53 (m, 2H), 4.46 (d, *J* = 8.5 Hz, 1H), 3.88 (m, 1H), 2.16−2.01 (m, 1H), 1.97−1.86 (m, 1H), 1.01 (m, 3H). MS (ESI) *m/z*: $[M - H]^-$ calcd for $C_{17}H_{20}BrN_2O_7P_2^-$ 504.99; found 505.2.

*[(S)-Chloro[hydroxy({[(1R)-1-phenylpropyl]amino})phosphoryl]-methyl][(2-nitrophenyl)methoxy]phosphinic Acid **23a** and Its (R) Isomer **23b**.* Using general method 2, the α-chloro methylene-(bisphosphonate) derivative **19a/b** as bis(triethylammonium) salts (0.3 mmol) in DMSO (1.0 mL) was reacted with (*R*)-1-phenylpropan-1-amine **21** (403 μL, 2.8 mmol), 2,2′-dithiodipyridine (198 mg, 0.9 mmol), and triphenylphosphine (236 mg, 0.9 mmol) for 16 h at rt. Purification was performed on a SAX column, isocratic mode, using 10% acetonitrile in 0.5 M triethylammonium bicarbonate, pH 7.9, at a flow rate of 8.0 mL/min and UV detection (280 nm); the title compounds **23a/b** eluted at 7 min. After removal of solvent under reduced pressure, separation of the diastereomers was performed using preparative RP-C18 HPLC, isocratic mode, using 32% acetonitrile in 0.1 M triethylammonium carbonate, pH 8.5, at a flow rate of 8.0 mL/min and UV detection (280 nm). Total (isomer ratio 1:1) yield 38% (by NMR).

The fast diastereomer, (*R*)-CHCl-(*R*)-auxiliary **23b** was eluted at 9.2 min: ^{31}P NMR (202 MHz, CD_3OD): δ 12.57 (d, *J* = 5 Hz, 1P), 11.82 (d, *J* = 5 Hz, 1P). ^{1}H NMR (500 MHz, CD_3OD): δ 8.01 (m, 1H), 7.89 (m, 1H), 7.64 (m, 1H), 7.48−7.38 (m, 1H), 7.35−7.30 (m, 2H), 7.25−7.19 (m, 2H), 7.15−7.09 (m, 1H), 5.52−5.37 (m, 2H), 4.26 (m, 1H), 4.69 (m, 1H), 2.03−1.76 (m, 2H), 0.76 (t, *J* = 7.2 Hz, 3H).

The slow diastereomer, (*S*)-CHCl-(*R*)-auxiliary **23a** was eluted at 10 min: ^{31}P NMR (202 MHz, CD_3OD): δ 10.90 (d, *J* = 4 Hz, 1P), 10.40 (d, *J* = 4.0 Hz, 1P).

*[(S)-Fluoro[hydroxy({[(1R)-1-phenylpropyl]amino})phosphoryl]-methyl][(2-nitrophenyl)methoxy]phosphinic Acid **24a** and Its (R) Isomer **24b**.* Using general method 2, the corresponding bisphosphonate derivative **20a/b** (1.0 g, 1.6 mmol) in anhydrous DMF (50 mL) was reacted with (*R*)-1-phenylpropan-1-amine **21** (863 μL, 6.0 mmol), 2,2′-dithiodipyridine (441 mg, 2.0 mmol), and triphenylphosphine (525 mg, 2.0 mmol) for 3 h at rt. After completion, the resulting mixture was diluted with water and solvent was removed under reduced pressure. The residue was purified on preparative RP-C18 HPLC, in isocratic mode, using 27% acetonitrile in 0.1 M triethylammonium carbonate, pH 8.5, at a flow rate of 8.0 mL/min and UV detection (280 nm). Total (isomer ratio 1:1) yield 56% (by NMR).

The slow diastereomer, (*S*)-CHF-(*R*)-auxiliary **24a**, was eluted at 27.7 min: ^{19}F NMR (376 MHz, CD_3OD): δ −218.67 (ddd, *J* = 60,

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52.5, 45 Hz). ^{31}P NMR (162 MHz, CD_3OD): δ 10.50 (dd, J = 60, 12 Hz, 1P), 9.75 (dd, J = 60, 12 Hz, 1P). ^{1}H NMR (400 MHz, CD_3OD): δ 8.06 (m, 1H), 7.71 (m, 1H), 7.68−7.60 (m, 1H), 7.49 (m, 1H), 7.39−7.33 (m, 2H), 7.24 (m, 2H), 7.16−7.09 (m, 1H), 5.40 (m, 2H), 4.59 (m, 1H), 4.27 (m, 1H), 1.91−1.64 (m, 2H), 0.81 (m, 3H). MS (ESI) m/z: $[M - H]^-$ calcd for $C_{17}H_{20}FN_2O_7P_2^-$ 445.1; found 445.3.

The fast diastereomer, (*R*)-CHF-(*R*)-auxiliary **24b**, was eluted at 25.4 min: ^{19}F NMR (470 MHz, CD_3OD): δ −218.87 (app. bm). ^{31}P NMR (202 MHz, CD_3OD): δ 10.50 (dd, J = 60.5, 11 Hz), 9.70 (dd, J = 60.5, 11 Hz). ^{1}H NMR (500 MHz, CD_3OD): δ 7.84−7.75 (m, 2H), 7.43 (m, 1H), 7.26−7.17 (m, 1H), 7.05 (m, 2H), 7.00−6.91 (m, 2H), 6.86 (m, 1H), 5.13 (m, 2H), 4.47−4.24 (m, 1H), 3.99 (m, 1H), 1.61−1.38 (m, 2H), 0.59−0.47 (m, 3H). MS (ESI) m/z: $[M - H]^-$ calcd for $C_{17}H_{20}FN_2O_7P_2^-$ 445.1; found 445.2.

Removal of the Chiral Auxiliary. General Method 3. Separated diastereomers (**22a**, **22b**, **23a**, **23b**, **24a**, and **24b**) were treated with aqueous HCl (1 M) at rt for 2−16 h. The cleavage was monitored by MS. After completion, the reaction mixture was concentrated under reduced pressure. Residual HCl was coevaporated multiple times with water and methanol. The triacids products were obtained by passage through a pipet column of DOWEX H^+ using a mixture of MeOH/water (1:1) as eluent.

*[(S)-Bromo({hydroxy[(2-nitrophenyl)methoxy]phosphoryl})-methyl]phosphonic Acid **25a** and Its (R) Isomer **25b**.* Using general method 3, 50 mg of each stereoisomer **22a** or **22b** was dissolved in 2 mL of aqueous HCl (1 M) *(pH was not adjusted)* and was stirred for 16 h. The α-bromo methylenebis(phosphonic acid) derivatives **25a** and **25b** were obtained as a colorless film (quantitative yield).

The slow isomer, (*S*)-CHBr **25a**: MS (ESI) m/z: $[M - H]^-$ calcd for $C_8H_9BrNO_8P_2^-$ 387.9; found 388.2.

The fast isomer, (*R*)-CHBr **25b**: ^{31}P NMR (202 MHz, CD_3OD): δ 12.84 (b, 1P), 10.96 (b, 1P). ^{1}H NMR (500 MHz, CD_3OD): δ 8.04 (m, 1H), 7.79 (m, 1H), 7.67 (m, 1H), 7.43 (m, 1H), 5.33 (m, 2H), 3.92 (t, J = 15.6 Hz, 1H). MS (ESI) m/z: $[M - H]^-$ calcd for $C_8H_9BrNO_8P_2^-$ 387.9; found 388.2.

*[(S)-Chloro({hydroxy[(2-nitrophenyl)methoxy]phosphoryl})-methyl]phosphonic Acid **26a** and Its (R) Isomer **26b**.* Using general method 3, 50 mg of each stereoisomer **23a** or **23b** was dissolved in 1 mL of aqueous HCl (1 M) *(pH was not adjusted)* and stirred for 16 h. The α-chloro methylenebis(phosphonic acids) derivative **26a** and **26b** were obtained in solid form (quantitative yield).

The slow isomer, (*S*)-CHCl **26a**: ^{1}H NMR (500 MHz, CD_3OD): δ 8.14 (m, 1H), 8.03 (m, 1H), 7.77 (m, 1H), 7.61−7.53 (m, 1H), 5.58 (m, 2H), 4.20 (t, J = 16.8 Hz, 1H). MS (ESI) m/z: $[M - H]^-$ calcd for $C_8H_9ClNO_8P_2^-$ 343.94; found 344.0.

The fast isomer, (*R*)-CHCl **26b**: ^{1}H NMR (500 MHz, CD_3OD): δ 8.16 (m, 1H), 8.06−7.92 (m, 1H), 7.78 (m, 1H), 7.57 (m, 1H), 5.62 (m, 2H), 4.29 (t, J = 17.2 Hz, 1H). MS (ESI) m/z: $[M - H]^-$ calcd for $C_8H_9ClNO_8P_2^-$ 343.94; found 344.1.

*[(S)-Fluoro({hydroxy[(2-nitrophenyl)methoxy]phosphoryl})-methyl]phosphonic Acid **27a** and Its (R) Isomer **27b**.* Using general method 3, 50 mg of each stereoisomer **24a** or **24b** was dissolved in 2 mL of aqueous HCl (1 M) and stirred for 3 h. The α-fluoro methylenebis(phosphonic acids) derivative **27a** and **27b** were obtained in solid form (quantitative yield).

The slow isomer, (*S*)-CHF **27a**: ^{19}F NMR (470 MHz, D_2O, pH 10): δ −221.96 (ddd, J = 61, 52, 42 Hz). ^{31}P NMR (202 MHz, D_2O, pH 10): δ 10.17 (bd, J = 61 Hz, 1P), 8.89 (bd, J = 61 Hz, 1P). ^{1}H NMR (500 MHz, D_2O): δ 7.65−7.57 (m, 1H), 7.42−7.33 (m, 1H), 7.26 (m, 1H), 7.08−6.97 (m, 1H), 4.94−4.82 (m, 2H), 4.39 (dt, J = 45.3, 12.6 Hz, 1H). MS (ESI) m/z: $[M - H]^-$ calcd for $C_8H_9FNO_8P_2^-$ 328.0; found 328.1.

The fast isomer, (*R*)-CHF **27b**: ^{19}F NMR (376 MHz, CD_3OD): δ −227.65 (ddd, J = 64, 55, 45 Hz). ^{31}P NMR (162 MHz, D_2O, pH 10): δ 11.10 (dd, J = 64, 14.5 Hz, 1P), 9.05 (dd, J = 55, 14.5 Hz, 1P). ^{1}H NMR (400 MHz, CD_3OD): δ 8.15 (m, 1H), 7.94 (m, 1H), 7.80−7.73 (m, 1H), 7.61−7.53 (m, 1H), 5.59 (m, 2H), 5.15 (app. dt, J = 46, 13.5 Hz, 1H). MS (ESI) m/z: $[M - H]^-$ calcd for $C_8H_9FNO_8P_2^-$ 328.0; found 328.1.

Coupling with Activated dCMP and Subsequent Removal of Photoreactive Group to Obtain (*R*)- or (*S*)-β,γ-CHX-dCTP (X = Br, Cl, F) Analogues 33a, 33b, 34a, 34b, 35a, and 35b. General Method 4. To a solution of the bisphosphonic acid derivative **25a**, **25b**, **26a**, **26b**, **27a**, and **27b** (1.0 equiv) in a mixture of EtOH/H_2O (1:1) [0.13 M] was added $(Bu)_3N$ to adjust the pH to 2.5−3.0, and the mixture stirred for 15 min. Solvent was removed under reduced pressure, and the residue was coevaporated 3 times with anhydrous DMF. To this residue **25′a**, **25′b**, **26′a**, **26′b**, **27′a**, and **27′b** was added a solution of 2 equiv of 2′-deoxycytidine 5′-phosphoromorpholidate[52] **29** in anhydrous DMSO [0.1 M]. The solution stirred at rt for 2 to 5 d and the reaction was monitored by ^{31}P NMR. After completion, the crude material was purified by preparative SAX HPLC, gradient mode, at a flow rate of 8.0 mL/min and UV detection (280 nm), with A/H_2O and B/0.5 M triethylammonium bicarbonate pH 7.5, 0−10 min, A/100%, 10−16 min, B/0%-B/55%, 16−25 min, B/100%. The products **30a**, **30b**, **31a**, **31b**, **32a**, and **32b** were diluted in water, transferred to a quartz cuvette and irradiated at 365 nm for 2 d with a UVP lamp stand (model: UVLS-28; power source 115 V, 60 Hz, 0.16 A) at 10 cm distance. Deprotection was monitored by ^{31}P NMR. After completion, the resulting reddish-brown solution was purified by RP-C18 HPLC, isocratic mode, using acetonitrile 4% in 0.1 M triethylammonium bicarbonate, pH 7−8, at a flow rate of 8.0 mL/min and UV detection (280 nm). The yields were determined by UV.

*[(R)-{[({[(2R,5R)-5-(4-Amino-2-oxo-1,2-dihydropyrimidin-1-yl)-3-hydroxyoxolan-2-yl]methoxy}(hydroxy)phosphoryl)oxy](hydroxy)-phosphoryl}(bromo)methyl]phosphonic Acid and Its (S) Isomer, (R)-β,γ-CHBr-dCTP **33a** and (S)-β,γ-CHBr-dCTP **33b**.* Using general method 4, the (*S*) **33b** and (*R*) **33a** diastereomers were obtained as triethylammonium salts in 7% yields.

(*R*)-β,γ-CHBr-dCTP **33a**: ^{31}P NMR (162 MHz, D_2O, pH 11.6): δ 7.57 (d, J = 5 Hz, 1P), 5.76 (dd, J = 27.5, 5 Hz, 1P), −11.25 (dd, J = 27.5, 5 Hz, 1P). ^{1}H NMR (400 MHz, D_2O): δ 7.86 (d, J = 7.5 Hz, 1H), 6.20 (t, J = 6.6 Hz, 1H), 6.01 (d, J = 7.5 Hz, 1H), 4.53 (m, 1H), 4.16−4.03 (m, 3H), 3.69 (m, 1H), 2.32−2.14 (m, 2H). HRMS (ESI-TOF) m/z: $[M - H]^-$ calcd for $C_{10}H_{16}BrN_3O_{12}P_3^-$ 541.9130; found 541.9130. Characterization data are consistent with the literature (stereoisomer pair).[29]

(*S*)-β,γ-CHBr-dCTP **33b**: ^{31}P NMR (243 MHz, D_2O, pH 10.1): δ 7.95 (d, J = 5 Hz, 1P), 6.26 (dd, J = 26.5, 5 Hz, 1P), −11.08 (d, J = 26.5 Hz, 1P). ^{1}H NMR (400 MHz, D_2O) 7.84 (d, J = 7.5 Hz, 1H), 6.20 (t, J = 6.7 Hz, 1H), 6.02 (d, J = 7.5 Hz, 1H), 4.49 (b, 1H), 4.10 (b, 3H), 3.81 (m, 1H), 2.34−2.14 (m, 2H). HRMS (ESI-TOF) m/z: $[M - H]^-$ calcd for $C_{10}H_{16}BrN_3O_{12}P_3^-$ 541.9130; found 541.9130. Characterization data are consistent with the literature (stereoisomer pair).[29]

*[(R)-{[({[(2R,5R)-5-(4-Amino-2-oxo-1,2-dihydropyrimidin-1-yl)-3-hydroxyoxolan-2-yl]methoxy}(hydroxy)phosphoryl)oxy](hydroxy)-phosphoryl}(chloro)methyl]phosphonic Acid and Its (S) Isomer, (R)-β,γ-CHCl-dCTP **34a** and (S)-β,γ-CHCl-dCTP **34b**.* Using general method 4, the (*S*) **34b** and (*R*) **34a** diastereomers were obtained as triethylammonium salts in 18% and 9% yield, respectively.

(*R*)-β,γ-CHCl-dCTP **34a**: ^{31}P NMR (162 MHz, D_2O, pH 10): δ 9.70 (d, J = 26.5 Hz,1P), 2.79 (bd, J = 26.5 Hz, 1P), −11.26 (d, J = 26.5 Hz, 1P). ^{1}H NMR (400 MHz, D_2O): δ 7.92 (d, J = 7.5 Hz, 1H), 6.19 (t, J = 6.5 Hz, 1H), 6.07 (d, J = 7.5 Hz, 1H), 4.56−4.44 (m, 1H), 4.18−4.03 (m, 3H), 3.89 (m, 1H), 2.40−2.14 (m, 2H). HRMS (ESI-TOF) m/z: $[M - H]^-$ calcd for $C_{10}H_{16}ClN_3O_{12}P_3^-$ 497.9635; found 497.9641. Characterization data are consistent with the literature (stereoisomer pair).[29]

(*S*)-β,γ-CHCl-dCTP **34b**: ^{31}P NMR (162 MHz, D_2O, pH 10): δ 9.70 (d, J = 26.5 Hz, 1P), 3.19−2.23 (m, 1P), −11.28 (d, J = 26.5 Hz, 1P). ^{1}H NMR (500 MHz, D_2O): δ 7.93 (d, J = 7.5 Hz, 1H), 6.20 (t, J = 6.5 Hz, 1H), 6.08 (d, J = 7.5 Hz, 1H), 4.56−4.45 (m, 1H), 4.23−4.03 (m, 3H), 3.90 (m, 1H), 2.37−2.15 (m, 2H). HRMS (ESI-TOF) m/z: $[M - H]^-$ calcd for $C_{10}H_{16}ClN_3O_{12}P_3^-$ 497.9635; found 497.9627. Crystal structure available at PDB entry 6BEM.[28] Characterization data are consistent with the literature (stereoisomer pair).[29]

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*[(R)-{[({[(2R,5R)-5-(4-Amino-2-oxo-1,2-dihydropyrimidin-1-yl)-3-hydroxyoxolan-2-yl]methoxy}(hydroxy)phosphoryl)oxy](hydroxy)-phosphoryl}(fluoro)methyl]phosphonic Acid and Its (S) Isomer, (R)-β,γ-CHF-dCTP **35a** and (S)-β,γ-CHF-dCTP **35b**.* Using general method 4, and after an additional analytical SAX HPLC purification (1.0 mL/min, 280 nm), gradient mode, A/H_2O and B/4% acetonitrile in 0.3 M triethylammonium bicarbonate, pH 7.5 buffer: 0 to 20 min, A/100%, 20−40 min, B/0%−100%, the (*S*) **35b** and (*R*) **35a** diastereomers were obtained as triethylammonium salts in 6% and 27% yields, respectively.

(*R*)-β,γ-CHF-dCTP **35a**: ^{19}F NMR (564 MHz, D_2O, pH 10): δ −216.93 (ddd, *J* = 65, 62, 56 Hz). ^{31}P NMR (243 MHz, D_2O, pH 10): δ 7.11 (dd, *J* = 56, 14.5 Hz, 1P), 4.89 (ddd, *J* = 65, 27, 14.5 Hz, 1P), −10.89 (d, *J* = 27 Hz, 1P). ^{1}H NMR (400 MHz, D_2O): δ 7.82 (d, *J* = 7.5 Hz, 1H), 6.19 (t, *J* = 6.5 Hz, 1H), 6.00 (d, *J* = 7.5 Hz, 1H), 4.74 (m, 1H), 4.56−4.35 (m, 1H), 4.05 (m, 3H), 2.44−2.23 (m, 1H), 2.22−2.09 (m, 1H). HRMS (ESI-TOF) *m*/*z*: [M − H]$^-$ calcd for $C_{10}H_{16}FN_3O_{12}P_3^-$ 481.9931; found 481.9933. Crystal structure available at PDB entry 6BEL.[28] Characterization data are consistent with the literature (stereoisomer pair).[29]

(*S*)-β,γ-CHF-dCTP **35b**: ^{19}F NMR (564 MHz, D_2O, pH 10): δ −216.75 (ddd, *J* = 60, 51, 45 Hz). ^{31}P NMR (243 MHz, D_2O, pH 10): δ 7.02 (dd, *J* = 51, 14.5 Hz, 1P), 4.79 (ddd, *J* = 60, 30, 14.5 Hz, 1P), −10.98 (d, *J* = 30 Hz, 1P). ^{1}H NMR (400 MHz, D_2O): δ 7.84 (d, *J* = 7.5 Hz, 1H), 6.20 (t, *J* = 6.5 Hz, 1H), 6.01 (d, *J* = 7.5 Hz, 1H), 4.74 (m, 1H), 4.55−4.45 (m, 1H), 4.23−3.88 (m, 3H), 2.38−2.25 (m, 1H), 2.23−2.08 (m, 1H). HRMS (ESI-TOF) *m*/*z*: [M − H]$^-$ calcd for $C_{10}H_{16}FN_3O_{12}P_3^-$ 481.9931; found 481.9941. Characterization data are consistent with the literature (stereoisomer pair).[29]

Synthesis of Individual Diastereomers of β,γ-CH(CH_3)-dATP Analogues 44a and 44b. Introduction of the 2-Nitrobenzyl Group 17. *(1-{Hydroxy[(2-nitrophenyl)methoxy]phosphoryl}ethyl)-phosphonic Acid **37a/b**.* A solution of (1-phosphonoethyl)-phosphonic acid **36** (1.7 mmol), 2-nitrobenzyl bromide **17** (1.5 equiv, 2.55 mmol), and DIEA (8 equiv, 13.6 mmol) in anhydrous DMF (20 mL) was heated at 90 °C for 4 h. Completion of the reaction was monitored by ^{31}P NMR each hour. After 4 h, solvent was removed under reduced pressure, and the residue was dissolved in ethyl acetate (25 mL) and the target compound was extracted with water (3 × 25 mL). The solvent was removed under reduced pressure and the crude mixture was purified by preparative RP-C18 HPLC, isocratic mode, using 15% acetonitrile in 0.1 M triethylammonium bicarbonate, pH 7.5 (8.0 mL/min, 280 nm) (rt = 12 min). After removal of solvents under reduced pressure and coevaporation with DMF, the desired product **37a/b** was obtained as bis-(triethylammonium) salt (493 mg, 55%). Pale yellow oil: ^{31}P NMR (202 MHz, CD_3OD): δ 22.47 (b, 1P), 20.65 (b, 1P). ^{1}H NMR (600 MHz, CD_3OD): δ 8.14 (d, *J* = 8.1 Hz, 1H), 8.07 (d, *J* = 8.7 Hz, 1H), 7.72 (t, *J* = 7.7 Hz, 1H), 7.46 (t, *J* = 7.9 Hz, 1H), 5.49−5.39 (m, 2H), 2.22−2.09 (m, 1H), 1.51−1.42 (m, 3H). ^{1}H NMR (500 MHz, D_2O): δ 8.03 (d, *J* = 8.2 Hz, 1H), 7.86 (d, *J* = 7.9 Hz, 1H), 7.67 (t, *J* = 7.6 Hz, 1H), 7.42 (t, *J* = 7.9 Hz, 1H), 5.26−5.14 (m, 2H), 1.91 (ddq, *J* = 22.1, 14.9, 7.5 Hz, 1H), 1.19 (ddd, *J* = 17.2, 14.8, 7.6 Hz, 3H). MS (ESI) *m*/*z*: [M − H]$^-$ calcd for $C_9H_{12}NO_8P_2^-$ 324.00; found 324.079.

Introduction of the Chiral Auxiliary 38. *[(1S)-1-[Hydroxy-({[(1R)-2-methoxy-2-oxo-1-phenylethyl]amino})phosphoryl]ethyl]-[(2-nitrophenyl)methoxy]phosphinic Acid **39b** and Its (S) Isomer **39a**.* The corresponding bisphosphonate derivative **37a/b** in bis(triethylammonium) salt form (0.285 mmol) was lyophilized and dissolved in anhydrous pyridine (1.0 mL). Methyl (*R*)-(−)-phenylglycinate **38** hydrochloride (1.5 equiv, 0.427 mmol) was coevaporated with anhydrous DMF (3 × 2 mL) and added to the bisphosphonate derivative mixture **37a/b** followed by addition of anhydrous TEA (2 equiv, 0.57 mmol). The mixture was stirred at 50 °C under a nitrogen atmosphere for 5−10 min. In a separate flask, a mixture of 2,2′-dithiodipyridine (1.2 equiv, 0.342 mmol) and triphenylphosphine (1.2 eqiuv, 0.342 mmol) was dissolved in anhydrous pyridine and stirred for 10−15 min to give a light-yellow solution. This solution was then added to the bisphosphonate derivative **37a/b** solution and stirred at 50 °C for 4 h. The completion of the reaction was monitored by MS and ^{31}P NMR and the reaction was quenched by 0.25 M ammonium acetate solution. The crude material was purified by dual-pass preparative HPLC: preparative SAX HPLC (8.0 mL/min, 280 nm) gradient mode with A/10% acetonitrile in H_2O and B/0.5 M triethylammonium bicarbonate pH 8.0 buffer: 0−7.5 min A/100%, 7.5−12 min B/0%-B/55%, 12−16 min B/55%, 16−18 min B/55%-B/100%, and 18−25 min B/100% (rt = 17.43 min), followed by preparative RP-C18 HPLC (8.0 mL/min, 280 nm) isocratic mode with 25.5% acetonitrile in 0.25 M ammonium acetate, pH 6.5.

The fast diastereomer, (*S*)-$CHCH_3$-(*R*)-auxiliary, **39a** was eluted at 13.58 min and after evaporation of volatiles under reduced pressure, a colorless film was obtained as ammonium salts (56.0 mg, 55%, NMR). ^{31}P NMR (202 MHz, D_2O, pH 10.0): δ 23.60 (d, *J* = 2.1 Hz, 1P), 20.92 (d, *J* = 2.1 Hz, 1P). ^{1}H NMR (500 MHz, CD_3OD): δ 8.13 (d, *J* = 8.1 Hz, 1H), 7.88 (d, *J* = 7.9 Hz, 1H), 7.76 (t, *J* = 7.7 Hz, 1H), 7.57 (t, *J* = 7.8 Hz, 1H), 7.48−7.13 (m, 5H), 5.29−5.10 (m, 2H), 5.05 (d, *J* = 8.0 Hz, 1H), 3.68 (s, 3H), 1.27 (td, *J* = 16.3, 7.2 Hz, 3H). MS (ESI) *m*/*z*: [M − H]$^-$ calcd for $C_{18}H_{21}N_2O_9P_2^-$ 471.07, found: 471.132.

The slow diastereomer, (*R*)-$CHCH_3$-(*R*)-auxiliary **39b** was eluted at 14.43 min and further purified by preparative RP-C18 HPLC to avoid cross contamination. A colorless film was obtained as ammonium salts (49.7 mg, 51.7%, NMR). ^{31}P NMR (202 MHz, D_2O, pH 10): δ 23.83 (b, 1P), 21.03 (b, 1P). ^{1}H NMR (500 MHz, D_2O): δ 7.88 (d, *J* = 7.8 Hz, 1H), 7.61 (d, J = 7.8 Hz, 1H), 7.51 (dd, J = 7.7 Hz, 1H), 7.29 (dd, *J* = 7.8 Hz, 1H), 7.12−7.03 (m, 5H), 3.36 (s, 3H), 2.92 (ddd, *J* = 9.8, 7.3, 6.0 Hz, 2H), 1.06−0.98 (m, 3H). MS (ESI) *m*/*z*: [M − H]$^-$ calcd for $C_{18}H_{21}N_2O_9P_2^-$ 471.07, found: 471.0.

Removal of the Chiral Auxiliary. *[(1R)-1-{Hydroxy[(2-nitrophenyl)methoxy]phosphoryl}ethyl]phosphonic Acid **40b** and Its (S) Isomer **40a**.* 15 mg of each stereoisomer **39a** or **39b** was dissolved in 2 mL of aqueous HCl (pH = 1) and stirred for 3 h. The completion of reaction was monitored by ^{31}P NMR. After completion, the reaction mixture was concentrated under reduced pressure. Residual HCl was coevaporated multiple times with water and methanol. Purification was performed on DOWEX H^+ using a mixture of methanol/water (1:1) as eluent. Bisphosphonic acid derivatives **40a**, **40b** were obtained as a colorless film (quantitative yield).

The fast isomer, (*S*)-$CHCH_3$ **40a**: ^{31}P NMR (202 MHz, D_2O, pH 10): δ 27.87 (d, *J* = 2.3 Hz, 1P), 16.63 (d, *J* = 2.3 Hz, 1P). ^{1}H NMR (400 MHz, CD_3OD): δ 8.14 (dd, *J* = 8.2, 1.2 Hz, 1H), 7.97 (dd, *J* = 7.9, 1.3 Hz, 1H), 7.76 (td, *J* = 7.6, 1.3 Hz, 1H), 7.62−7.51 (m, 1H), 5.53 (d, *J* = 7.2 Hz, 2H), 2.63−2.35 (m, 1H), 1.51 (m, 3H). HRMS (ESI-TOF) *m*/*z*: [M − H]$^-$ calcd for $C_9H_{12}NO_8P_2^-$ 324.0038; found 324.0035.

The slow isomer, (*R*)-$CHCH_3$ **40b**: ^{31}P NMR (202 MHz, D_2O, pH 10): δ 23.60 (b, 1P), 20.24 (b, 1P). ^{1}H NMR (500 MHz, D_2O): δ 8.08−8.01 (m, 1H), 7.81−7.72 (m, 1H), 7.70−7.63 (m, 1H), 7.49−7.42 (m, 1H), 5.30 (d, *J* = 7.6 Hz, 2H), 2.34 (m, 1H), 1.30 (m, 3H). HRMS (ESI-TOF) *m*/*z*: [M − H]$^-$ calcd for $C_9H_{12}NO_8P_2^-$ 324.0038; found 324.0034.

Coupling with Activated dAMP 42. *{[(2R,5R)-5-(6-Amino-9H-purin-9-yl)-3-hydroxyoxolan-2-yl]methoxy}({hydroxy[(1S)-1-{hydroxy[(2-nitrophenyl)methoxy]phosphoryl}ethyl]phosphoryl}-oxy)phosphinic Acid **43b** and Its (R) Isomer **43a**.* 5′-dAMP as the free acid (1 equiv) was dissolved in acetonitrile [0.3 M] containing 12 equiv of TEA and cooled to 0 °C. Then, trifluoroacetic anhydride (TFAA) (15 equiv) in acetonitrile [1.2 M] cooled to 0 °C in an ice-bath was added dropwise by a gastight syringe to the dAMP solution under dry N_2 and the reaction allowed to stir for 10 min at rt. Excess TFAA was removed under reduced pressure and the residue cooled to 0 °C. Then a cold (ice bath) solution of *N*-methylimidazole (15 equiv) in anhydrous acetonitrile [3 M] containing TEA (2.5 equiv) was added dropwise under dry N_2. After 10 min, the reaction was complete (^{31}P NMR, δ −11.54 ppm (in CD_3CN)). The product, dAMP-*N*-methylimidazolide[39,64] **42** was used for coupling reaction. In a separate flask, a solution of (1-phosphonoethyl)phosphonic acid derivative **40a** or **40b** (∼6−7 mg, either isomer) in 2 mL of EtOH/H_2O (1:1) was adjusted to pH 8 by adding $(Bu)_4NOH$ (40% in water) and stirred for 15 min. Solvent was removed under reduced

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pressure and the residue **40′a** or **40′b** was coevaporated (3 × 3 mL) with anhydrous DMF and cooled to 0 °C in an ice-bath. The prepared activated dAMP-*N*-methylimidazolide[32,56] **42** was added dropwise to the prepared solution of bisphosphonate derivative **40′a** or **40′b** in an ice-bath under N_2. The solution was stirred at rt for 4 h and the reaction was monitored by ^{31}P NMR. After completion, the reaction mixture was dissolved in 2 mL DCM and the desired product was extracted by water (3 × 3 mL) and purified by dual-pass preparative HPLC: preparative SAX HPLC (8.0 mL/min, 259 nm) gradient mode with A/H_2O and B/0.5 M triethylammonium bicarbonate, pH 7.5: 0−7.5 min B/0−55%, 7.5−15 min B/55%, 15−20 min B/55−100%, 20−25 min B/100% followed by preparative RP-C18 HPLC (8.0 mL/min, 259 nm) isocratic mode with 15% acetonitrile in 0.1 M triethylammonium bicarbonate, pH 7.5, to yield the title compounds **43a**, **43b** (70−75%) as triethylammonium salts.

The fast isomer, (*R*)-CHCH$_3$ **43a** (SAX: rt = 14.67 min, C18: rt = 11.27 min) (0.012 mmol): ^{31}P NMR (202 MHz, D_2O, pH 10): δ 22.37 (b, 1P), 12.90 (d, J = 29.0 Hz, 1P), −11.01 (d, J = 29.0 Hz, 1P). ^{1}H NMR (500 MHz, D_2O): δ 8.26 (s, 1H), 8.02 (s, 1H), 7.83 (d, J = 8.2, 1H), 7.70 (d, J = 7.8 Hz, 1H), 7.48 (ddd, J = 7.6, 1.3 Hz, 1H), 7.20 (dd, J = 7.8, 1H), 6.23 (t, J = 6.5 Hz, 1H), 5.10 (d, J = 8.0 Hz, 1H), 4.13−4.00 (m, 3H), 2.55 (dd, J = 13.5, 6.6 Hz, 1H), 2.42−2.35 (m, 1H), 2.24 (ddq, J = 23.0, 7.5 Hz, 0H), 1.28 (dt, J = 16.9, 7.5 Hz, 3H). MS (ESI) m/z: [M − H]$^-$ calcd for $C_{19}H_{24}N_6O_{13}P_3^-$ 637.06; found 637.1.

The slow isomer, (*S*)-CHCH$_3$ **43b** (SAX: rt = 13.23 min, C18: rt = 13.11 min), (0.011 mmol): ^{31}P NMR (202 MHz, D_2O, pH 10): δ 22.46 (b, 1P), 12.91 (d, J = 29.1 Hz, 1P), −10.97 (d, J = 29.1 Hz, 1P). ^{1}H NMR (500 MHz, D_2O): δ 8.27 (s, 1H), 8.03 (s, 1H), 7.84 (d, J = 8.2, 1H), 7.70 (d, J = 8.2 Hz, 1H), 7.48 (ddd, J = 7.6, 1.3 Hz, 1H), 7.19 (dd, J = 7.6, 1H), 6.22 (t, J = 6.5 Hz, 1H), 5.13 (d, J = 8.0 Hz, 2H), 4.10−4.04 (m, 3H), 2.59−2.46 (m, 1H), 2.46−2.31 (m, 1H), 2.24 (ddq, J = 22.9, 15.3, 7.5 Hz, 1H), 1.27 (dt, J = 16.9, 7.5 Hz 3H). MS (ESI) m/z: [M − H]$^-$ calcd for $C_{19}H_{24}N_6O_{13}P_3^-$ 637.06; found 637.1.

Removal of Photoreactive Group to Obtain (*R*)- or (*S*)-β,γ-CH(CH$_3$)-dATP Analogues 44a, 44b. *[(1S)-1-{[{[{[(2R,5R)-5-(6-Amino-9H-purin-9-yl)-3-hydroxyoxolan-2-yl]methoxy}(hydroxy)-phosphoryl)oxy](hydroxy)phosphoryl}ethyl]phosphonic Acid and Its (R) Isomer, (S)-β,γ-CH(CH$_3$)-dATP* ***44b*** *and (R)-β,γ-CH(CH$_3$)-dATP* ***44a***. The protected triphosphate analogue was diluted in water, introduced in a quartz cuvette, and irradiated at 365 nm for 1 day with a UVP Lamp Stand (model: UVLS-28; power source 115 V, 60 Hz, 0.16 A) at 10 cm distance. The reaction was monitored by ^{31}P NMR. After completion, the resulting reddish-brown solution was purified by RP-C18 HPLC (8.0 mL/min, 258 nm), isocratic mode, using 6.5% acetonitrile in 0.1 M triethylammonium bicarbonate, pH 7.5 to yield the individual title compounds (42%) as triethylammonium salts. The yields were determined by UV.

The fast isomer, (*R*)-β,γ-CH(CH$_3$)-dATP **44a** eluted at 16.94 min; (0.00504 mmol): ^{31}P NMR (202 MHz, D_2O, pH 8): δ 17.70 (d, J = 28.9 Hz, 1P), 17.13 (b, 1P), −10.93 (d, J = 29.8 Hz, 1P). ^{1}H NMR (500 MHz, D_2O): δ 8.39 (s, 1H), 8.13 (s, 1H), 6.40 (t, J = 6.8 Hz, 1H), 4.16 (b, 1H), 4.13−4.05 (m, 1H), 4.05−3.96 (m, 1H), 2.75−2.62 (m, 1H), 2.53−2.40 (m, 1H), 2.04 (m, 1H), 1.24 (dt, J = 17.63, 7.5 Hz, 3H). HRMS (ESI-TOF) m/z: [M − H]$^-$ calcd for $C_{12}H_{19}N_5O_{11}P_3^-$ 502.0294; found 502.0291.

The slow isomer, (*S*)-β,γ-CH(CH$_3$)-dATP **44b** eluted at 16.96 min (0.0046 mmol): ^{31}P NMR (243 MHz, D_2O, pH 8): δ 18.76 (d, J = 30.2 Hz), 16.22 (b, 1P), −10.88 (d, J = 30.4 Hz). ^{1}H NMR (600 MHz, D_2O): δ 8.37 (s, 1H), 8.11 (s, 1H), 6.38 (t, J = 6.8 Hz, 1H), 4.16−4.12 (m, 1H), 4.10−4.03 (m, 1H), 4.01−3.94 (m, 1H), 2.50−2.41 (m, 1H), 2.05−1.89 (m, 1H), 1.19 (ddd, J = 18.1, 16.9, 7.4 Hz, 3H). HRMS (ESI-TOF) m/z: [M − H]$^-$ calcd for $C_{12}H_{19}N_5O_{11}P_3^-$ 502.0294; found 502.0298.

Synthesis of Individual Diastereomers of β,γ-CHX-dATP (X = F, Cl) Analogues 12a, 12b, 13a, and 13b. Introduction of the Chiral Auxiliary (*R*)-3 or (*S*)-3. General Method 5. The chiral auxiliary (*R*)- or (*S*)-methyl mandelate (***R***)-**3** or (***S***)-**3** was introduced into the α-halo methylenebis(phosphonate) derivatives **1** or **2** using a Mitsunobu condensation.[35] The manual silica gel column chromatography used in our prior work[35] was replaced here by automated flash column chromatography (UV detector) using gradient elution (ethyl acetate: 0%−100% in hexane). The (*S*)-methyl mandelate was not used in our initial report,[35] here we coupled the CHF-bisphosphonate synthon **2** with (*S*)-methyl mandelate (***S***)-**3** to investigate the effect of the auxiliary chirality on the RP-C18 HPLC elution order (see the Stereochemistry section).

Methyl (2R)-2-({[[(Dimethoxyphosphoryl)(fluoro)methyl](methoxy)phosphoryl}oxy)-2-phenylacetate ***5c/d***. According to general method 5, 2.1 mmol of methyl [(dimethoxyphosphoryl)(fluoro)methyl]phosphonate **2** was coupled to (*S*)-methyl mandelate (***S***)-**3** giving 766 mg (95%) of desired product **5c**/**d**. Colorless oil. ^{19}F NMR (470 MHz, CD_3OD): δ −229.74 to −233.32 (m). ^{31}P NMR (202 MHz, CD_3OD): δ 13.93−12.31 (m). ^{1}H NMR (500 MHz, CD_3OD): δ 7.54−7.38 (m, 5H), 6.06−5.92 (m, 1H), 5.84−5.46 (m, 1H), 4.18−3.66 (m, 12H). Data for using (***R***)-**3** auxiliary:[35] ^{31}P NMR (202 MHz, CD_3OD): δ 14−12 (m).

Methyl (2S)-2-({[[Chloro(dimethoxyphosphoryl)methyl](methoxy)phosphoryl}oxy)-2-phenylacetate ***4a/b***. According to general method 5, 2.4 mmol of methyl [chloro(dimethoxyphosphoryl)methyl]phosphonate **1** was coupled with methyl (*R*)-methyl mandelate (***R***)-**3** to yield 913.55 mg (95%) of the desired product as a yellow oil. ^{31}P NMR (202 MHz, $CDCl_3$): δ 18.31−17.10 (m); Characterization data are consistent with the literature.[35] NMR (202 MHz, $CDCl_3$): δ 16−15 (m).

Deprotection of the Chiral Synthons. General Method 6. Following our previously reported method,[35] the appropriate trimethyl methylenebisphosphonate methyl mandelate ester **4a**/**b**, **5c**/**d** was tridemethylated using BTMS,[81] then the reaction mixture was stirred overnight at pH 8.3 (added $NaHCO_3$) to hydrolyze the methyl ester of mandelate. The crude product was converted to the acid form by passage through DOWEX H^+ and used in the next step without further purification. A small amount of the bisphosphonate side product from complete de-esterification was removed during chiral synthon separation by HPLC.

(2R)-2-({[Fluoro(phosphono)methyl](hydroxy)phosphoryl}oxy)-2-phenylacetic Acid ***5′c/d***. Using general method 6, 146 mg (379 μmol) of the α-fluoro methylenebis(phosphonate) mandelate ester **5c**/**d** gave 97.0 mg (78%) of the title compound **5′c**/**d** as a colorless oil. A mixture of two diastereomers (1:1) is evident in the NMR spectra: ^{19}F NMR (470 MHz, CD_3OD): δ −228.48 to −229.17 (m). ^{31}P NMR (162 MHz, D_2O, pH 10): δ 13.09 (δ = 0.30, 2 × dd/overlap, J = 62, 49 Hz, 1P), 7.79 ($\Delta\delta$ = 0.34, 2 × dd/overlap, J = 54.8, 13.6 Hz, 1P). ^{1}H NMR (500 MHz, D_2O): δ 7.49−7.19 (m, 5H), 5.40 (m, 1H). MS (ESI) m/z: [M − H]$^-$ calcd for mono methyl ester $C_{10}H_{12}FO_8P_2^-$ 340.99, found: 341.15. Characterization data are consistent with the literature (opposite enantiomer).[35]

(2S)-2-({[Chloro(phosphono)methyl](hydroxy)phosphoryl}oxy)-2-phenylacetic Acid ***4′a/b***. Using general method 6, 758 mg (1.9 mmol) of the α-chloro methylenebis(phosphonate) mandelate ester **4a**/**b** gave 497.2 mg (1.51 mmol, 80%) of the title compounds **4′a**/**b** as a pale yelow oil. A mixture of two diastereomers (1:1) is evident in the NMR spectra: ^{31}P NMR (202 MHz, D_2O, pH 10.0): δ 14.79 (d, J = 5 Hz, 1P), 14.10 (d, J = 6 Hz, 1P), 9.08 (d, J = 5 Hz, 1P), 8.99 (d, J = 6 Hz, 1P), MS (ESI) m/z: [M − H]$^-$ calcd for monomethyl ester $C_{10}H_{12}ClO_8P_2^-$ 356.97, found: 357.2. Characterization data are consistent with the literature.[35]

Preparation of the Separable Chiral Synthons. General Method 7. Following our previously reported method,[35] dimorpholidate derivatives of α-halo bisphosphonates **6a**/**b**, **7c**/**d** were synthesized and separated by RP-HPLC. Then, the bisphosphonate morpholine group was removed by addition of HCl (aq.) and passage through DOWEX H^+. However, HPLC conditions used for separation of the chiral synthons were modified to increase yield and separation (up to 40 mg of the reaction mixture per run). The chiral synthons were separated by preparative RP HPLC using a Phenomenex Luna C18(2) HPLC column (5 μm, 250 mm × 21 mm) with 14−15% acetonitrile in 0.2 M ammonium acetate (NH_4OAc) buffer, pH 6.5, at a flow rate of 8.0 mL/min and a UV detection (256 nm). Our

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original[35] chiral synthon separation method with 0.1 M triethylammonium bicarbonate buffer allowed to inject only 4 mg of diastereomer mixture per run. Moreover, the retention time is reduced from 28 to 14 min.

[(S)-Fluoro({hydroxy[(1R)-2-(morpholin-4-yl)-2-oxo-1-phenylethoxy]phosphoryl})methyl]phosphonic Acid 7′c and Its (R) Isomer 7′d. According to general method 7, 95.5 mg (0.29 mmol) of α-fluoro methylenebis(phosphonic acid) mandelate ester **5′c/d** was reacted with morpholine and the mixture of isomers **7c/d** was separated by RP HPLC using a Phenomenex Luna C18(2) HPLC column (5 μm, 250 mm × 21 mm) with 15% acetonitrile in 0.2 M ammonium acetate (NH_4OAc) buffer, pH 6.5, at a flow rate of 8.0 mL/min and UV detection (256 nm).

The fast diastereomer, (*S*)-CHF-(*R*)-auxiliary **7c** eluted at 12 min and 46.7 mg (69% by NMR) as a colorless film was obtained as ammonium salts after solvent removal. ^{19}F NMR (564 MHz, D_2O, pH 10.0): δ −218.79 (app. td, 61, 45.5 Hz). ^{31}P NMR (243 MHz, D_2O, pH 10.0): δ 9.76 (dd, *J* = 61, 12 Hz, 1P), 9.68 (dd, *J* = 61, 12 Hz, 1P). ^1H NMR (600 MHz, D_2O): δ 7.34−7.28 (m, 5H), 5.97 (m, 1H), 4.52−4.46 (m,1H), 3.57−3.17 (m, 12H), 2.91 (m, 4H). MS (ESI) *m/z*: $[M - H]^-$ calcd for $C_{17}H_{24}FN_2O_8P_2^-$ 465.10, found: 465.24. Characterization data are consistent with the literature (opposite enantiomer).[35]

The slow diastereomer, (*R*)-CHF-(*R*)-auxiliary **7d** was eluted at 13 min and 51.3 mg (75.8% by NMR) as a colorless film was obtained as ammonium salts after solvent removal. ^{19}F NMR (376 MHz, D_2O, pH 10.0): δ −218.56 (app. td, *J* = 61, 45.5 Hz). ^{31}P NMR (243 MHz, D_2O, pH 10.0): δ 10.21 (dd, *J* = 61, 12.3 Hz, 1P), 9.75 (dd, *J* = 61, 12.3 Hz, 1P). ^1H NMR (600 MHz, D_2O): δ 7.40−7.28 (m, 5H), 6.03−5.97 (m, 1H), 4.80−4.70 (m, 1H), 3.63−3.37 (m, 12H), 2.97 (m, 4H). MS (ESI) *m/z*: $[M - H]^-$ calcd for $C_{17}H_{24}FN_2O_8P_2^-$ 465.10, found: 465.16. Characterization data are consistent with the literature (opposite enantiomer).[35]

Conversion of the α-fluoro methylenebis(phosphonate) dimorpholidates **7c**, **7d** to the corresponding monomorpholidate triacids **7′c**, **7′d** was effected by adjusting the solution pH to 2 with HCl, stirring for 30 min and passage through DOWEX H^+. After evaporation of volatiles, a colorless film was obtained. MS (ESI) *m/z*: $[M - H]^-$ calcd for $C_{13}H_{17}FNO_8P_2^-$ 396.04, found: 396.34, **7′c**.

[(R)-Chloro({hydroxy[(1S)-2-(morpholin-4-yl)-2-oxo-1-phenylethoxy]phosphoryl})methyl]phosphonic Acid 6′a and Its (S) Isomer 6′b. According to general method 7, 250 mg (0.7 mmol) of α-chloro methylenebis(phosphonic acid) mandelate ester **4′a/b** was reacted with excess morpholine and the mixture of isomers **6a/b** was separated by RP HPLC using a Phenomenex Luna C18(2) HPLC column (5 μm, 250 mm × 21 mm) with 14% acetonitrile in 0.2 M ammonium acetate (NH_4OAc) buffer, pH 6.5, at a flow rate of 8.0 mL/min with a UV detection (256 nm).

The fast diastereomer, (*R*)-CHCl-(*S*)-auxiliary **6b** eluted at 16 min and 146 mg (83% by NMR) as a colorless film was obtained as ammonium salts after solvent removal. ^{31}P NMR (202 MHz, D_2O, pH 10.0): δ 11.08 (d, *J* = 5.3 Hz, 1P), 11.03 (d, *J* = 5.3 Hz, 1P). ^1H NMR (500 MHz, CD_3OD): δ 7.55 (d, *J* = 7.5 Hz, 2H), 7.42−7.29 (m, 3H), 6.20 (d, *J* = 8.9 Hz, 1H), 3.82 (t, *J* = 15.0 Hz, 1H), 3.59 (m, 10H), 3.45 (m, 2H), 3.19 (m, 4H). MS (ESI) *m/z*: $[M - H]^-$ calcd for $C_{17}H_{24}ClN_2O_8P_2^-$ 481.07, found: 481.38. Characterization data are consistent with the literature.[35]

The slow diastereomer, (*S*)-CHCl-(*S*)-auxiliary **6a** eluted at 18 min and 140 mg (81% by NMR) as a colorless film was obtained as ammonium salts after solvent removal. ^{31}P NMR (202 MHz, D_2O, pH 10.0): δ 11.70 (d, *J* = 4.5 Hz, 1P), 10.92 (d, *J* = 4.5 Hz, 1P). ^1H NMR (500 MHz, D_2O): δ 7.42 (d, *J* = 7.3 Hz, 2H), 7.40−7.31 (m, 3H), 6.03 (d, *J* = 8.6 Hz, 1H), 3.68 (t, *J* = 15.3 Hz, 1H), 3.57−3.40 (m, 12H), 3.26 (d, *J* = 27.9 Hz, 4H). MS (ESI) *m/z*: $[M - H]^-$ calcd for $C_{17}H_{24}ClN_2O_8P_2^-$ 481.07, found: 481.12. Characterization data are consistent with the literature.[35]

Conversion of α-chloro methylenebis(phosphonate) dimorpholidates **6a**, **6b** to the corresponding monomorpholin triacids **6′a**, **6′b** was yielded by adjusting pH to 2 by HCl, stirring for 30 min at rt and passage through DOWEX H^+. After evaporation of volatiles, colorless film was obtained. MS (ESI) *m/z*: $[M - H]^-$ calcd for $C_{13}H_{17}ClNO_8P_2^-$ 412.01, found: 412.25, **6′b** and 412.47, **6′a**. Characterization data are consistent with the literature.[35]

Coupling of Chiral Synthons with Activated dAMP. General Method 8. Following our previously reported method,[35] individual α-halo diastereomers **6′a**, **6′b**, **7′c**, and **7′d** were coupled with 5′-dAMP-morpholidate[52] in DMSO following by dual HPLC purification: (1) Macherey-Nagel Nucleogel SAX 1000−10 (25 mm × 15 cm) preparative column, using a gradient (0−10 min, B/0%−60%; 10−16 min, B/60%; 16−25 min, B/60%−100%) of 0.5 M triethylammonium bicarbonate buffer, pH 7.4, at a flow rate of 8 mL/min; (2) RP HPLC using a Phenomenex Luna C18(2) column (5 μm, 250 mm × 21 mm) with 15% acetonitrile in 0.1 M triethylammonium bicarbonate, pH 7.5, at a flow rate of 8.0 mL/min with a UV detection (260 nm).

{[(2R,3R,5R)-5-(6-Amino-9H-purin-9-yl)-3-hydroxyoxolan-2-yl]-methoxy}({[(R)-fluoro({hydroxy[(1R)-2-(morpholin-4-yl)-2-oxo-1-phenylethoxy]phosphoryl})methyl](hydroxy)phosphoryl}oxy)-phosphinic Acid 9c and Its (S) Isomer 9d. Using general method 8, 20 mg (50.3 μmol) of the (*S*)-CHF bisphosphonic acid derivative **7′c** was coupled with 5′-dAMP-morpholidate, purified by HPLC, and dried under reduced pressure to yield 10.7 mg of the title compound **9c** as triethylammonium salts (31%, determined by UV). Colorless oil. ^{19}F NMR (564 MHz, D_2O, pH 10.0): δ −218.88 (app. td, *J* = 61, 46 Hz). ^{31}P NMR (243 MHz, D_2O, pH 10.0): δ 8.93 (dd, *J* = 61, 18 Hz, 1P), 0.99 to −0.38 (m, 1P), −11.00 (d, *J* = 26.5 Hz, 1P). ^1H NMR (600 MHz, D_2O): δ 8.29 (s, 1H), 8.10 (s, 1H), 7.28 (m, 3H), 7.24−7.18 (m, 2H), 6.33 (t, *J* = 6.9 Hz, 1H), 5.96 (d, *J* = 8.3 Hz, 1H), 4.85 (dt, *J* = 46, 13 Hz, 1H), 4.12 (m, 2H), 4.02−3.92 (m, 3H), 3.44 (m, 4H), 3.41−3.34 (m, 4H), 2.70−2.65 (m, 1H), 2.40 (m, 1H). MS (ESI) *m/z*: $[M - H]^-$ calcd for $C_{23}H_{29}FN_6O_{13}P_3^-$ 709.10, found: 709.39.

Also, 15.0 mg (37.8 μmol) of the (*R*)-CHF bisphosphonic acid derivative **7′d** was coupled with 5′-dAMP-morpholidate,[52] purified by HPLC, and dried under reduced pressure to yield 14.9 mg of the title compound **9d** as triethylammonium salts (28%, determined by UV). Colorless oil: ^{19}F NMR (564 MHz, D_2O, pH 10.0): δ −218.72 (app. td, *J* = 60, 46 Hz). ^{31}P NMR (243 MHz, D_2O, pH 10.0): δ 8.94 (dd, *J* = 60, 18.5 Hz), 0.30 (ddd, *J* = 60, 27, 18.5 Hz), −11.03 (d, *J* = 27 Hz). ^1H NMR (600 MHz, D_2O): δ 8.24 (s, 1H), 8.06 (s, 1H), 7.26−7.22 (m, 2H), 7.18−7.09 (m, 3H), 6.29 (t, *J* = 6.8 Hz, 1H), 5.94 (d, *J* = 8.2 Hz, 1H), δ 4.82 (dt, *J* = 46, 13 Hz, 1H), 4.10 (m, 1H), 3.98 (m, 2H), 3.57−3.51 (m, 1H), 3.51−3.44 (m, 1H), 3.43−3.30 (m, 6H), 2.55−2.49 (m, 1H), 2.40−2.33 (m, 1H). MS (ESI) *m/z*: $[M - H]^-$ calcd for $C_{23}H_{29}FN_6O_{13}P_3^-$ 709.10, found: 709.51.

{[(2R,3R,5R)-5-(6-Amino-9H-purin-9-yl)-3-hydroxyoxolan-2-yl]-methoxy}({[(S)-chloro({hydroxy[(1R)-2-(morpholin-4-yl)-2-oxo-1-phenylethoxy]phosphoryl})methyl](hydroxy)phosphoryl}oxy)-phosphinic Acid 10b and Its (R) Isomer 10a. Using general method 8, 25 mg (60.4 μmol) of the (*R*)-CHCl bisphosphonic acid derivative **6′b** was coupled with 5′-dAMP-morpholidate,[52] purified by HPLC, and dried under reduced pressure to yield 13.15 mg of the title compound **10b** as triethylammonium salts (30%, determined by UV). ^{31}P NMR (202 MHz, D_2O, pH 10.0): δ 10.17 (d, *J* = 9 Hz, 1P), 1.97 (dd, *J* = 26.5, 9 Hz, 1P), −11.10 (d, *J* = 26.5 Hz, 1P). ^1H NMR (500 MHz, D_2O): δ 8.34 (s, 1H), 8.11 (s, 1H), 7.39−7.34 (m, 2H), 7.32−7.22 (m, 3H), 6.37 (t, *J* = 6.8 Hz, 1H), 6.06 (d, *J* = 8.5 Hz, 1H), 4.15 (m, 1H), 4.04 (m, 2H), 3.94 (m, 1H), 3.55−3.38 (m, 8H), 3.03 (m, 1H), 1.13 (m, 2H). MS (ESI) *m/z*: $[M - H]^-$ calcd for $C_{23}H_{29}ClN_6O_{13}P_3^-$ 725.07, found: 725.48.

Also, 27 mg (65.3 μmol) of the (*S*)-CHCl bisphosphonic acid derivative **6′a** was coupled with 5′-dAMP-morpholidate, purified by HPLC, and dried under reduced pressure to yield 7.5 mg of the title compound **10a** as triethylammonium salts (28%, determined by UV). Colorless oil: ^{31}P NMR (162 MHz, D_2O, pH 10.0): δ 10.53 (d, *J* = 9 Hz, 1P), 2.05 (dd, *J* = 26.5, 9 Hz, 1P), −11.13 (d, *J* = 26.5 Hz, 1P). ^1H NMR (400 MHz, D_2O): δ 8.33 (s, 1H), 8.11 (s, 1H), 7.38−7.34 (m, 2H), 7.31−7.20 (m, 3H), 6.36 (t, *J* = 6.8 Hz, 1H), 6.04 (d, *J* = 8.2 Hz, 1H), 4.14 (m, 1H), 4.05 (m, 2H), 3.95 (m, 1H), 3.63−3.33 (m,

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8H), 3.32−3.20 (m, 1H), 2.94−2.83 (m, 1H). MS (ESI) m/z: $[M - H]^-$ calcd for $C_{23}H_{29}ClN_6O_{13}P_3^-$ 725.07, found: 725.68.

Synthesis of Deprotected β,γ-CHX-dATP (X = F, Cl) Analogues 12a, 12b, 13a, and 13b. General Method 9. Following our previously reported method,[35] the mandelate ester protecting group was removed using Pd/C hydrogenolysis (reaction monitored by ^{31}P NMR and MS). Purification was performed by preparative RP HPLC using a Phenomenex Luna C18(2) HPLC column (5 μm, 250 mm × 21 mm) with 6.5% acetonitrile in 0.1 M triethylammonium bicarbonate buffer, pH 7.5, at a flow rate of 8.0 mL/min with a UV detection (260 nm).

[(R)-{[{[{[(2R,5R)-5-(6-amino-9H-purin-9-yl)-3-hydroxyoxolan-2-yl]methoxy}(hydroxy)phosphoryl)oxy](hydroxy)phosphoryl}-(fluoro)methyl]phosphonic Acid and Its (S) Isomer, (R)-β,γ-CHF-dATP ***12a*** *and (S)-β,γ-CHF-dATP* ***12b****.* According to general method 8, both stereoisomers **9c**, **9d** were deprotected by hydrogenolysis and purified by preparative RP-C18 HPLC.

(*R*)-β,γ-CHF-dATP **12a** (obtained from **9c**) eluted at 10.5 min to yield 4.9 mg (65% by UV) of the title compound **12a** as triethylammonium salts. Colorless film. ^{19}F NMR (564 MHz, D_2O, pH 10.0): δ −217.12 (ddd, J = 65.5, 55.5, 45.5 Hz). ^{31}P NMR (243 MHz, D_2O, pH 10.0): δ 7.15 (dd, J = 55.5, 14.5 Hz, 1P), 5.06 (ddd, J = 65.5, 28, 14.5 Hz, 1P), −10.77 (d, J = 28 Hz, 1P). ^1H NMR (600 MHz, D_2O): δ 8.35 (s, 1H), 8.11 (s, 1H), 6.38 (t, J = 6.8 Hz, 1H), 4.77−4.64 (m, 1H), 4.15 (m, 1H), 4.05 (m, 1H), 3.99 (m, 1H), 2.68 (m, 1H), 2.45 (m, 1H). HRMS (ESI-TOF) m/z: $[M - H]^-$ calcd for $C_{11}H_{16}FN_5O_{11}P_3^-$ 506.0043; found 506.0037.

(*S*)-β,γ-CHF-dATP **12b** (obtained from **9d**) eluted at 9.9 min to yield 4.1 mg (77% by UV) of the title compound **12b** as triethylammonium salts. Colorless film. ^{19}F NMR (564 MHz, D_2O, pH 10.0): δ −216.98 (ddd, J = 65.5, 55.5, 45.5 Hz). ^{31}P NMR (202 MHz, D_2O, pH 10.0): δ 6.91 (dd, J = 55.5, 15 Hz, 1P), 4.75 (ddd, J = 65.5, 28, 15 Hz, 1P), −10.98 (d, J = 28 Hz, 1P). ^1H NMR (600 MHz, D_2O): δ 8.34 (s, 1H), 8.11 (s, 1H), 6.33 (t, J = 6.8 Hz, 1H), 4.73 (m, 1H), 4.12 (m, 1H), 4.06 (m, 1H), 3.97 (m, 1H), 2.63 (m, 1H), 2.44 (m, 1H). HRMS (ESI-TOF) m/z: $[M - H]^-$ calcd for $C_{11}H_{16}FN_5O_{11}P_3^-$ 506.0043; found 506.0044.

[(S)-{[{[{[(2R,5R)-5-(6-Amino-9H-purin-9-yl)-3-hydroxyoxolan-2-yl]methoxy}(hydroxy)phosphoryl)oxy](hydroxy)phosphoryl}-(chloro)methyl]phosphonic Acid and Its (S) Isome, (R)-β,γ-CHCl-dATP ***13a*** *and (S)-β,γ-CHCl-dATP* ***13b****.* According to general method 9, both stereoisomers **10a**, **10b** were deprotected by hydrogenolysis and purified by preparative RP HPLC using a Phenomenex Luna C18(2) HPLC column (5 μm, 250 mm × 21 mm) with 5.5% acetonitrile in 0.1 M triethylammonium bicarbonate buffer, pH 7.4, at a flow rate of 8.0 mL/min with a UV detection (260 nm). Hydrogenolysis of the CHCl-triphosphate analogues **10a**, **10b** was monitored carefully by MS to avoid reduction of CHCl group to CH_2.

(*S*)-β,γ-CHCl-dATP **13b** (obtained from **10b**) eluted at 23.1 min to yield 4.6 mg (58% by UV) of the title compound **13b** as triethylammonium salts. Colorless film. ^{31}P NMR (202 MHz, D_2O, pH 10.0): δ 8.92 (d, J = 7 Hz, 1P), 7.31 (dt, J = 26.5, 7 Hz, 1P), −10.65 (dd, J = 26.5, 5.5 Hz, 1P). ^1H NMR (500 MHz, D_2O): δ 8.34 (s, 1H), 8.10 (s, 1H), 6.37 (t, J = 6.8 Hz, 1H), 4.17 (s, 1H), 4.09−4.00 (m, 1H), 3.76 (m, 1H), 2.68 (m, 1H), 2.46 (m, 1H). HRMS (ESI-TOF) m/z: $[M - H]^-$ calcd for $C_{11}H_{16}ClN_5O_{11}P_3^-$ 521.9748, found: 521.9749.

(*R*)-β,γ-CHCl-dATP **13a** (obtained from **10a**) eluted at 23.0 min to yield 4.3 mg (78% by UV) of the title compound **13a** as triethylammonium salts. Colorless film. ^{31}P NMR (202 MHz, D_2O, pH 10.0): δ 8.62 (d, J = 6.5 Hz, 1P), 6.91 (d, J = 27, 6.5 Hz, 1P), −10.87 (d, J = 27 Hz, 1P). ^1H NMR (500 MHz, D_2O): δ 8.38 (s, 1H), δ 8.13 (s, 1H), 6.40 (t, J = 6.8 Hz, 1H), 4.18 (m, 1H), 4.12 (m, 1H), 4.03 (m, 1H), 3.77 (m, 1H), 2.71 (m, 2H), 2.47 (m, 1H). ^{13}C NMR (101 MHz, D_2O): δ 154.56 (C6), 151.46 (C8), 148.40 (C4), 140.17 (C2), 118.32 (C5), 85.72 (C4′), 83.62 (C1′), 71.05 (C3′), 65.24 (C5′), 48.43 (β,γ-CHCl, m), 39.05 (C2′). HRMS (ESI-TOF) m/z: $[M - H]^-$ calcd for $C_{11}H_{16}ClN_5O_{11}P_3^-$ 521.9748, found: 521.9749.

Synthesis of Individual Diastereomers of β,γ-CHCl-dTTP Analogues 11a, 11b. Coupling of Chiral Synthons to Activated dTMP. *5′-O-[({[(S)-Chloro{hydroxy[(1S)-2-(morpholin-4-yl)-2-oxo-1-phenylethoxy]phosphoryl}methyl](hydroxy)-phosphoryl}oxy)(hydroxy)phosphoryl]-2′-deoxythymidine* ***8b*** *and Its (R) Isomer* ***8a****.* Using general method 8, 0.021 mmol of the (*R*) **6′b** or (*S*)-CHCl bisphosphonic acid derivative **6′a** were coupled with 5′-dTMP-morpholidate,[32] purified by HPLC, and dried under reduced pressure.

(*S*)-Isomer **8b** (obtained from **6′b**): ^{31}P NMR (202 MHz, D_2O, pH 10): δ 10.14 (d, J = 9 Hz, 1P), 1.83 (dd, J = 27, J = 9 Hz, 1P), −11.36 (d, J = 27 Hz, 1P). ^1H NMR (500 MHz, D_2O): δ 7.67 (m, 1H), 7.56−7.43 (m, 5H), 6.39 (t, J = 7 Hz, 1H), 6.24 (d, J = 8 Hz, 1H), 4.64−4.62 (m, 1H), 4.19−4.17 (m, 3H), 4.10 (dd, J = 17 Hz, J = 16 Hz, 1H), 3.74−3.26 (m, 8H), 2.34−2.31 (m, 2H), 1.91 (s, 3H). MS (ESI) m/z: $[M - H]^-$ calcd for $C_{23}H_{30}ClN_3O_{15}P_3^-$ 716.1, found 715.8.

(*R*) isomer **8a** (obtained from **6′a**): ^{31}P NMR (202 MHz, D_2O, pH 10): δ 10.59 (d, J = 9 Hz, 1P), 2.04 (dd, J = 26, J = 9 Hz, 1P), −11.27 (d, J = 26 Hz, 1P). ^1H NMR (500 MHz, D_2O): δ 7.63 (s, 1H), 7.56−7.44 (m, 5H), 6.39 (t, J = 7 Hz, 1H), 6.22 (d, J = 8 Hz, 1H), 4.64−4.62 (m, 1H), 4.23−4.08 (m, 4H), 3.78−3.39 (m, 8H), 2.33−2.31 (m, 2H), 1.89 (s, 3H). MS (ESI) m/z: $[M - H]^-$ calcd for $C_{23}H_{30}ClN_3O_{15}P_3^-$ 716.1, found 716.0.

Synthesis of Deprotected β,γ-CHCl-dTTP Analogues 11a, 11b. *5′-O-[({[(S)-Chloro(phosphono)methyl](hydroxy)phosphoryl}-oxy)(hydroxy)phosphoryl]-2′-deoxythymidine and Its (R)-Isomer, (S)-β,γ-CHCl-dTTP* ***11b****, and (R)-β,γ-CHCl-dTTP* ***11a****.* According to general method 9, both stereoisomers **8a**, **8b** were deprotected by hydrogenolysis and purified by preparative RP HPLC using a Phenomenex Luna C18(2) HPLC column (5 μm, 250 mm × 21 mm) with 4.5% acetonitrile in 0.1 M triethylammonium bicarbonate buffer, pH 7.4, at a flow rate of 8.0 mL/min with a UV detection (260 nm). Hydrogenolysis of CHCl-triphosphate analogues **8a**, **8b** was monitored carefully by MS to avoid reduction of CHCl group to CH_2. Diastereomers **11a**, **11b** were obtained as triethylammonium salts (colorless film) in 13% and 16% yield (UV, 4 steps) respectively.

(*S*)-β,γ-CHCl-dTTP **11b**: ^{31}P NMR (202 MHz, D_2O, pH 10): δ 8.59 (b, 1P), 6.79 (bd, J = 27 Hz, 1P), −11.12 (d, J = 27 Hz, 1P). ^1H NMR (500 MHz, D_2O): δ 7.70 (m, 1H), 6.40 (t, J = 7 Hz, 1H), 4.69−4.66 (m, 1H), 4.26−4.19 (m, 3H), 3.92 (dd, J = 16 Hz, J = 15.5 Hz, 1H), 2.41−2.32 (m, 2H), 1.93 (s, 3H). MS (ESI) m/z: $[M - H]^-$ calcd for $C_{11}H_{17}ClN_2O_{13}P_3^-$ 513.0, found 513.0. Crystal structure available at PDB entry 6G2Q.[28] Characterization data are consistent with the literature (stereoisomer pair).[27]

(*R*)-β,γ-CHCl-dTTP **11a**: ^{31}P NMR (202 MHz, D_2O, pH 10): δ 8.58 (d, J = 6.5 Hz, 1P), 6.76 (dd, J = 27.5, J = 6.5 Hz, 1P), −11.10 (d, J = 27.5 Hz, 1P). ^1H NMR (500 MHz, D_2O): δ 7.68 (m, 1H), 6.40 (t, J = 7.0 Hz, 1H), 4.69−4.67 (m, 1H), 4.28−4.18 (m, 3H), 3.91 (dd, J = 16.5, J = 15 Hz, 1H), 2.41−2.31 (m, 2H), 1.93 (s, 3H). MS (ESI) m/z: $[M - H]^-$ calcd for $C_{11}H_{17}ClN_2O_{13}P_3^-$ 513.0, found 513.0. Crystal structure available at PDB entry 6CTM.[28] Characterization data are consistent with the literature (stereoisomer pair).[27]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.joc.0c01204.

Spectroscopic and other compound characterization data (^1H, ^{19}F, ^{31}P, ^{13}C NMR, COSY, HSQCAD, HRMS and LRMS), J value calculations, dipole moments/log D value calculations, and tabulation of bisphosphonic acid acid−base titration data (PDF)

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Notes

The authors declare no competing financial interest.

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