Application of Phosphoramidate Pronucleotide Technology to Abacavir Leads to a Significant Enhancement of Antiviral Potency

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We report the first application of pronucleotide (ProTide) technology to the antiviral agent abacavir (Ziagen), used for the treatment of HIV infection. The phenylmethoxyalaninyl phosphoramidate of abacavir was prepared in good yield in one step. Also prepared was the corresponding phosphoramidate of the guanine nucleoside analogue "carbovir". The antiviral profile of each of the parent nucleosides was compared to that of the phosphoramidate ProTides. A significant (28- to 60-fold) increase in anti-HIV potency was noted for the ProTide of abacavir but not for that of carbovir. These findings were in agreement with the markedly higher (ca. 37-fold) levels of carbovir triphosphate that are formed in CEM cells upon response to the abacavir ProTide compared with the parent abacavir compound. In contrast the anti-HBV potency of both abacavir and carbovir were improved (10- and 20-fold, respectively) by ProTide formation. As in CEM cells, the abacavir ProTide provided significantly enhanced carbovir triphosphate levels in HepG2 2.2.15 cells over that of the parent nucleoside. On the basis of these data, a series of phosphoramidate analogues with structural variation in the ester and amino acid regions were prepared and their antiviral profiles described. In addition, the pharmacokinetic disposition of the abacavir phenylethoxyalaninyl phosphoramidate was evaluated in Cynomolgus monkeys.

Introduction

Abacavir (**1**) (Scheme 1) is the first member of the well-studied carbocyclic nucleosides to be approved for use as a drug.[1−8] Carbocyclic nucleosides, lacking the labile glycosidic linkage between the heterocycle and sugar, have long been proposed to offer an attractive in vivo stability advantage over the 2′,3′-dideoxynucleosides. However, carbocyclic versions of 2′,3′-dideoxyadenosine (ddA), 2′,3′-dideoxyinosine (ddI), 2′,3′-dideoxycytidine (ddC), 2′,3′-dideoxyguanosine, and 3′-deoxythymidine have demonstrated only modest anti-HIV activity.[9] The anti-HIV activity of the racemic guanine member of this series, "carbovir" (CBV), **2**, was described by Vince and colleagues.[10] Although the triphosphate of (−)-carbovir is a potent and selective inhibitor of HIV reverse transcriptase (RT),[7,11] in vivo preclinical studies eliminated **2** as a candidate for anti-HIV therapy because of dose-limiting kidney and cardiac toxicities, poor oral bioavailability, and limited central nervous system (CNS) penetration.[8] The problems encountered with CBV could not be solved with existing prodrug strategies, e.g., 5′-aminoacylesters[12] or 2,6-diaminopurine prodrugs.[13] The realization of an unexpected enzymatic purine conversion/modification, possible only after formation of the monophosphate of a nucleoside, led to the understanding of the unique in vivo properties of abacavir and closely related analogues modified at the 6-position of the purine.[8] The contrasting in vivo profiles of abacavir and CBV are attributable to an activation pathway by which abacavir is converted to the triphosphate of CBV in lymphocytes. This pathway bypasses CBV, suggesting that the in vivo contrast is attributable to markedly differing exposures to the nucleoside CBV. Abacavir has demonstrated potent, selective anti-HIV activity in the clinic as a monotherapy and in combination with nucleoside reverse transcriptase inhibitors,[1−4] non-nucleoside reverse transcriptase inhibitors,[5] and protease inhibitors.[6]

Abacavir and a few other 6-modified purine analogues have exhibited anti-HIV activity comparable to that of carbovir against HIV-1 (IIIB) in MT-4 cells ($IC_{50} \approx 4$ μM).[8] This is approximately 4-fold more potent than ddI in this assay but less potent than AZT, 3TC, and ddC. The relative potency of the nucleoside RT inhibitors in the clinic does not parallel ranking from in vitro assays likely because of the many confounding variables such as pharmacokinetics and distribution of the nucleoside analogues and levels of active triphosphates in target cells and tissues. Indeed, 3TC and abacavir were selected for clinical evaluation largely because of their excellent safety and pharmacokinetic profiles in animals rather than superior in vitro potency relative to AZT. Metabolic studies have shown that abacavir is anabolized in infected and uninfected $CD4^+$ CEM cells and in uninfected peripheral blood lymphocytes (PBLs) to

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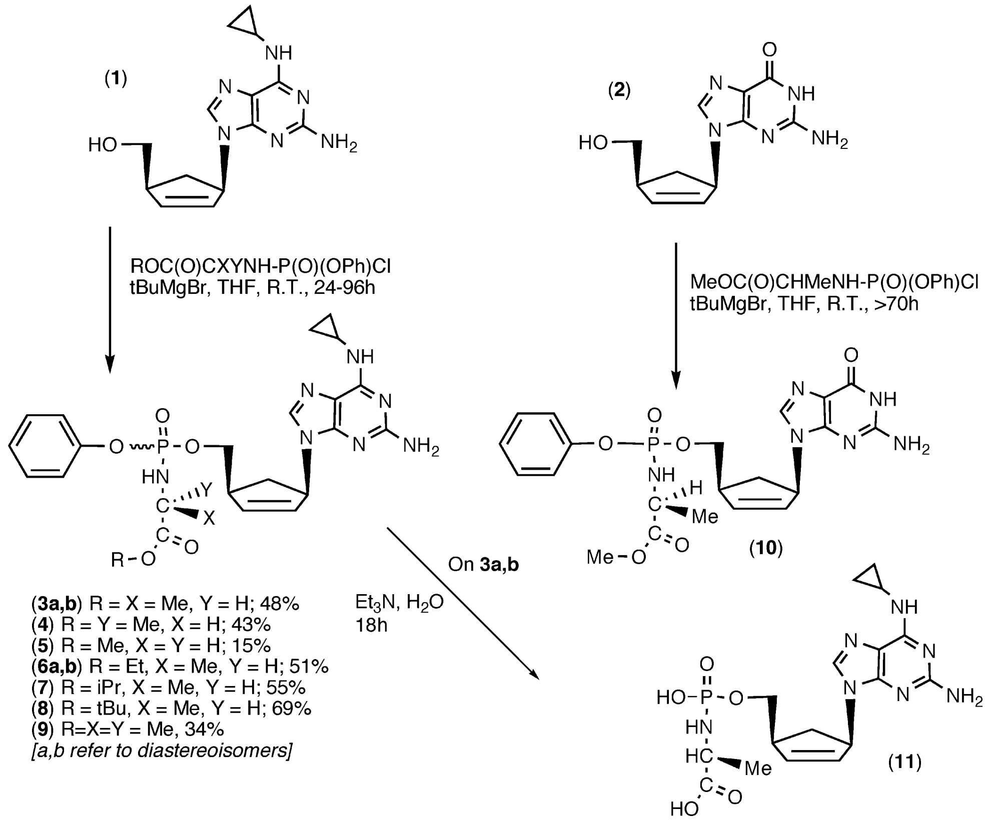
Scheme 1. Synthetic Route to Antiviral ProTides of Carbovir and Abacavir



(−)-CBV-TP, which is the only triphosphate observed.[8,14] Abacavir monophosphate was detected, but the di- and triphosphates of the nucleoside were not detected. The only radiolabeled di- and triphosphates detected were those of CBV. The levels of CBV-TP produced from abacavir were not significantly different from those formed from equimolar CBV, suggesting that the anabolic pathway from abacavir to CBV-TP is relatively efficient, although indirect. (−)-CBV-TP is a potent competitive inhibitor with respect to dGTP for RT-catalyzed DNA synthesis, with a K_i of 0.02 μM, and is selective over mammalian DNA polymerases.[8] The distinctive antiviral profile of abacavir, via its unique activation pathway, is attributable to two new enzymes: adenosine phosphotransferase, which phosphorylates abacavir to its' 5′-monophosphate (abacavir-MP), and a novel, previously unidentified and cytosolic enzyme "abacavir monophosphate deaminase", which deaminates abacavir-MP to CBV-MP.[14,15]

Since the phosphorylation of abacavir by adenosine phosphotransferase is unique to abacavir and little has been reported concerning the in vivo levels and distribution of this kinase, it was of interest to determine whether the potency of **1** against HIV could be improved via a pronucleotide (ProTide) approach[16] to achieve "kinase-bypass". In addition, it was of interest to determine whether the micromolar in vitro anti-HBV activity of abacavir, insufficient to provide any clinical indication of anti-HBV efficacy, could be improved after conversion to its ProTide forms.

Results and Discussion

Chemistry. The synthesis of phosphorylated derivatives of diaminopurine and guanine nucleosides is a challenge because of the highly polar nature of these compounds that limits their solubility in organic solvents and that also presents more than one possible site at which phosphorylation can occur. Previous work undertaken with the acyclic nucleoside analogue acyclovir investigated the use of N-protection as a means to increase the solubility of acyclovir and also to prevent N-phosphorylation on the guanine base.[17] However, these synthetic routes afford a lengthier synthesis, with N-protection, phosphorylation, and then deprotection, resulting in poor yields of the desired 5′-phosphoramidate derivative. Phosphorylation of unprotected acyclovir using phenylmethoxyalaninyl phosphorochloridate and *N*-methylimidazole (NMI) in THF resulted in little/no product being isolated. A variety of protecting groups (DMF, pixyl) have been utilized to N-protect the base and aid solubility in the phosphorylation reaction.[17] While these protected derivatives yielded the desired phosphoramidates, removal of protecting groups in the presence of the phosphoramidate proved to be troublesome because of basic cleavage of the ester. Similar problems were encountered in the synthesis of abacavir and other diaminopurine 5′-*O*-phosphoramidates. Thus, it was desirable to achieve a one-step conversion of diaminopurine and guanine nucleoside to 5′-phosphoramidates without the use of purine protecting groups.

The method of Uchiyama,[18] which claims to achieve selective O-phosphorylation without the need for N-protection of nucleosides, was investigated. This approach is based on the concept of *functional group activation* rather than protection. Thus, when a nucleoside is treated with 1 equiv of a strong organometalic base (such as *tert*-butylmagnesium chloride), an equilibrium mixture of the metal alkoxide and amide is formed. Selective O-phosphorylation depends on this equilibrium and the enhanced nucleophilicity of the oxygen function. Preferential formation of the magnesium iminoxide is observed with guanosine, but the magnesium alkoxide is more reactive, and the subsequent phosphorylated species equilibrate to form a single phosphorylated product, showing the desired 5′-O-phosphorylation.[18]

We chose to investigate if the Uchiyama method would be suitable for formation of the 5′-*O*-phosphoramidates of abacavir. Thus, treatment of abacavir with *tert*-butylmagnesium chloride in THF, followed by addition of phenylmethoxy-L-alaninyl phosphorochloridate, was observed to be very rapid and gave a good yield of desired products following silica gel purification. The reaction yielded a 1:1 mixture of diastereomers (**3a**,**b**) because of the chiral phosphorus. Other abacavir phosphoramidates (**4**−**9**) were prepared in a similar fashion. The phosphorylation of carbovir (**2**) as a suspension in THF under similar conditions was found to be more problematic, requiring greater excess of phenylmethoxy-alaninyl phosphorochloridate and longer reaction times to yield **10** (Scheme 1).

The chirality at the phosphorus center results in the formation of two diastereomeric phosphoramidate nucleosides in an approximately 1:1 ratio that were inseparable by silica gel chromatography. Because of the very potent antiviral activity of these mixtures (see below), we became interested in separating the diastereoisomers for comparison of antiviral activities. The diastereomeric mixtures of representative derivatives **3** and **6** were separated using supercritical fluid chromatography with a Chiralpak AS column and 22% methanol in carbon dioxide as eluent to give **3a**,**b** and **6a**,**b**. Data on these separated isomers are discussed below.

The metabolic intermediate (**11**) was efficiently prepared by hydrolysis of both phosphate and carboxylate esters of abacavir 5′-[phenyl(methoxy-L-alaninyl)]phosphate (**3**) in an aqueous solution of triethylamine. Purification of the concentrated reaction was conveniently carried out using a QMA solid-phase extraction cartridge (Sep-Pak Vac Accell Plus QMA cartridge, Waters Corp.) as an anion exchange chromatography column. Lyophilization of the eluent gave a diammonium salt that was unstable on storage for a few days at room temperature. However, passing the concentrated eluent through a Sep-Pak Vac Accell Plus CM cartridge (Na^+ form) followed by lyophilization gave a more stable disodium salt that could be stored at room temperature for several weeks.

For profiling in animals we chose to scale-up the ethyl ester derivative **6**. The procedure used to make multigram amounts **6** was modified from the process used to make research quantities, by using pyridine in addition to THF as a cosolvent. Use of pyridine allowed the reactions to be run more concentrated and reduced losses due to salt precipitation. Purification of the reaction by extraction followed by filtration through silica gel gave **6** as a white hygroscopic solid foam. Crystalline salt forms were sought, since the solid foams formed by the free bases trapped solvents and were difficult to formulate. As with abacavir, dibasic acids were found to crystallize the ProTides in a 1:1 complex.

Table 1. Stability and Solubility of Salts of **6**

salt form of **6**	solid type	solubility (mg/mL)[a]	solid-state stability[b]
free base	amorphous (hygroscopic)	0.054	93.7
glutarate	crystalline	0.084	98.9
fumarate	crystalline	0.086	97.1
succinate	crystalline	0.069	99.6

[a] Solubility in phosphate buffered saline (pH 7.4) at room temperature. [b] Solid-state stability is determined as the % of parent (AUC) after 2 weeks at 60 °C and ambient humidity normalized to initial AUC.

Thus, succinic acid, fumaric acid, and glutaric acid all gave crystalline products. Data on these salts are presented in Table 1. On the basis of the relative stability of these products, the succinate salt was chosen for pharmacokinetic evaluation. This abacavir phosphoramidate succinate salt was not deliquescent and was stable at 60 °C for over 2 weeks.

Antiviral Activity. The phenylmethoxy-L-alaninyl phosphoramidates of abacavir (**3**) and (−)-carbovir (**10**) were assessed for their antiviral activity with **1** and **2** as positive controls. Both carbovir and abacavir exhibited similar potencies and selectivities against HIV-1 and HIV-2, with EC_{50} values of 1.9−3 μM and CC_{50} values of 78−160 μM in CEM cell cultures (Table 2). The phenylmethoxy-L-alaninyl phosphoramidate derivative of carbovir (**10**) was similar in potency to carbovir with EC_{50} values approximately 2-fold lower than for carbovir in CEM cell cultures and 7-fold less potency in PBL. In contrast, the phenyl phosphoramidate derivative of abacavir (**3**) showed a 27-fold increase in potency against HIV-1 in the CEM cell line, a 25-fold increase in potency against HIV-1 in PBLs, and a 33-fold increase in potency against HIV-2 in the CEM cell cultures compared to abacavir. While compound **3** did show a decrease in its CC_{50}, the selectivity index (SI) was still more favorable compared to the parent drug. The differential behavior of carbovir and abacavir in human leukemic T cells upon derivatization is notable and surprising. This difference may be cell- or virus-dependent because ProTides **3** and **10** show similar enhancements of potency (ca. 10- to 20-fold) against HBV in Hep G2.2.15 cells. These data indicated to us that the SARs we have thoroughly established for d4T (stavudine) anti-HIV activity in lymphocytes may not fully apply to the guanine carbocyclic nucleosides, especially against HBV in hepatoma cells but also against MSV in C3H/3T3 cells and herpesviruses in HEL cell cultures (Table 3), where the ProTide **3** increased the antiviral activity of ABC (**1**) but where the ProTide **10** significantly *decreased* the antiviral activity of CBV (**2**).

Table 4 presents the anti-HIV profile of the amino acid and ester-modified series of abacavir phosphoramidates (**3**−**9**), compared to the parent abacavir (**1**). Against HIV, the amino acid dependence is strikingly similar to that we have noted for phosphoramidates of stavudine (d4T).[19] Thus, (L-)alanine emerges as the preferred amino acid. There is a clear stereochemical preference for L-amino acids, with a ca. 50-fold reduction in potency for D-alanine (**4**), this being rather similar to glycine (**5**) in potency. The effect of variation in the ester region (Table 4) also shows a close parallel to d4T phosphoramidate data.[20] Thus, the primary esters methyl and ethyl (**3**, **6**) were roughly equipotent. The

Table 2. Antiviral Activity for Abacavir, Carbovir, and Derivatives in Cell Culture[a]

	EC$_{50}$ (μM)				SI		EC$_{50}$ (μM)		EC$_{50}$ (μM)	
	HIV-1 (IIIB) CEM	HIV-1 PBL	HIV-2 (ROD) CEM	CC$_{50}$ (μM) CEM	HIV-1 CEM	HIV-2 CEM	MSV C3H/3T3	CC$_{50}$ (μM) C3H/3T3	HBV HepG2 2.2.15	CC$_{50}$ (μM) HepG22.215
3	0.07	0.008	0.09	13	190	150	1.6	>4	0.55	17
ABC (**1**)	1.9	0.20	3.0	78	41	26	8.8	>20	5.6	110
10	1.3	0.20	0.85	120	95	145	>20	>20	0.51	200
CBV (**2**)	2.0	0.03	2.3	160	79	68	6.0	>20	10	150

[a] PBL refers to peripheral blood lymphocytes. MSV: murine moloney sarcoma virus. EC$_{50}$: 50% effective concentration required to inhibit HSV-induced cytopathicity by 50%. CC$_{50}$: 50% cytostatic concentration required to inhibit HEL cell proliferation by 50%.

Table 3. Anti-HSV Activity for Carbovir and Abacavir Derivatives in HEL Cell Cultures[a]

	EC$_{50}$ (μM) for HSV TK$^+$		EC$_{50}$ (μM) for HSV-1 TK$^-$		
	HSV-1 (KOS)	HSV-2 (G)	B2006	VMW-1837	CC$_{50}$ (μM)
3	20	20	20	20	110
ABC (**1**)	900	900	900	320	200
10	480	480	>800	480	160
CBV (**2**)	5	8	50	13	15

[a] TK$^+$ refers to thymidine kinase-competent virus. TK$^-$ refers to thymidine kinase-deficient virus.

secondary/isopropyl (**7**) was slightly less potent and the *tert*-butyl (**8**) least potent, being ca. 100-fold less potent than the methyl parent compound (**3**). Against HBV, the most potent ProTide is that derived from the unusual achiral amino acid dimethylglycine (**9**), which was 295 times more potent than abacavir. The primary and secondary esters of the L-alanine analogues have similar activity and are approximately 10- to 15-fold more potent than abacavir. The *tert*-butyl ester of L-alanine has activity comparable to that of **1**. Dimethylglycine compound **9** is also very active against HIV, equivalent to the most potent L-Ala esters. The potency of the dimethylglycine analogue against HIV and HBV is interesting and unexpected, given the poor activity of the D-alanine analogues.

Table 5 contains a comparison of the two sets of purified phosphate isomers, compared to the succinate salts of the ~1:1 diastereomeric mixtures (**3a**,**b** and **6a**,**b**). In each case, the isomer that eluted from a reverse-phase column with liquid carbon dioxide/methanol with the shorter retention time proved to be more potent against HIV-1 (4- and 9-fold). However, against HBV the isomers were less differentiated. Since the diastereomeric mixtures were equiactive (within experimental deviation) with the more potent of the purified isomers, we decided to pursue the mixtures. We reasoned that the chirality at phosphorus is removed in the first step of conversion of the ProTide to monophosphate inside the cells (Scheme 2). Thus, on the basis of earlier metabolic studies with the analogues stavudine (d4T) phosphoramidates,[21] we reasoned that the activation intracellular route for the current compounds was likely to involve initial carboxyl ester hydrolysis to give a short-lived carboxylate intermediate, which would rapidly cyclize with loss of phenol to give another transient intermediate, a highly energetic five-membered anhydride, which would spontaneously hydrolyze to give the stable phosphate diester (**11**). In all prior metabolic studies, compounds of the general structure of **11** were frequently observed as major intracellular metabolites. A phosphoramidase-type activity is then implicated in the cleavage of the amino acid from (**11**) to generate abacavir monophosphate (ABCMP). This would then be processed as for abacavir via carbovir monophosphate (CBVMP) to the bioactive triphosphate. To further support this notion, we carried out some preliminary metabolism studies on radiolabeled **3**.[21]

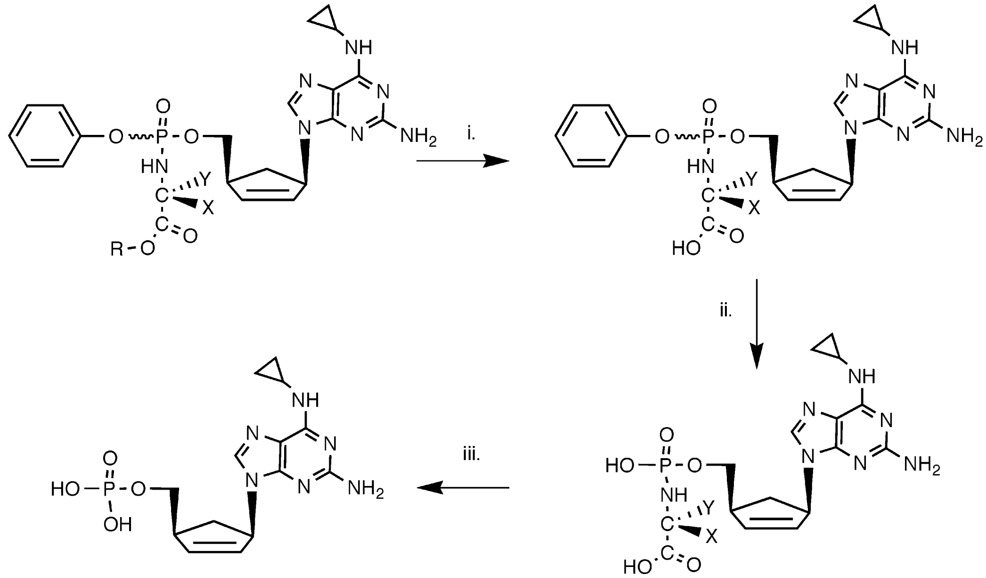
Anabolism in CEM and HepG2 2.215 Cells. Radiolabeled [^{3}H]abacavir and the aryloxymethylalaninyl ProTide of [^{3}H]abacavir monophosphate (**3**) were administered to CEM cell cultures at 10 μM. The highest levels of formed metabolites were recorded between 6 and 24 h of incubation.[22] ABC (**1**) was metabolized to its 5′-monophosphate ABCMP, and reasonable levels of carbovir 5′-phosphates, CBVMP, CBVDP, and CBVTP, were also detected in the cell extracts (Table 6). In fact, 82 pmol/10^9 CEM cells of CBVTP was formed after 24 h, which represents an ~0.1 μM CBVTP intracellular concentration (assuming that 10^9 CEM cells represent 0.8 μL of packed volume).[14] These levels were comparable with those reported earlier by Faletto et al. who found 160 pmol of CBVTP/10^9 CEM cells.[14] When radiolabeled **3** was administered to CEM cell cultures for 24 h, pronounced levels of alaninyl ABCMP (**11**) and detectable levels of ABCMP, CBVMP, CBVDP, and CBVTP were formed (Table 6). Interestingly, at 10 μM

Table 4. Antiviral Activity against HIV and HBV for a Series of Abacavir Phosphoramidates[a] with Varying Amino Acids and Esters

		EC$_{50}$ (μM)					EC$_{50}$ (μM)		
	amino acid/ester	HIV-1 CEM	HIV-2 CEM	CC$_{50}$ (μM) CEM	EC$_{50}$ (μM) HBV HepG2 2.2.15	CC$_{50}$ (μM) HepG2	HIV1 MT4	HIV2 MT4	CC$_{50}$ (μM) MT4
3a,**b**	L-Ala/Me	0.050	0.050	13	0.55	17	0.20	0.26	4.6
6	L-Ala/Ethyl	0.070	0.061	12	0.35	18	0.20	0.21	8.6
7	L-Ala /i-Pr	0.48	0.85	17	0.33	10	0.48	0.60	5.2
8	L-Ala/t-Bu	3.7	6.0	8.6	5.0	11	6.6	3.4	2.1
11	L-Ala "X"	1.2		115	1.9	110			
4	D-Ala/Me	1.4	4.5	46	1.3	52	3.4	4.1	4.5
5	Gly/Me	1.8	2	>56	1.0	58	7.2	5.4	8.5
9	DiMeGly/Me	0.067	0.064	>130	0.019	5.5	0.14	0.11	8.1
1		1.9	3.0	78	5.6	110	5.9	7.3	192
2		2.0	2.3	160	10.5	150			

[a] All compounds are ~1:1 mixtures of diastereomers (epimeric at phosphate). EC$_{50}$: 50% effective concentration required to inhibit HSV-induced cytopathicity by 50%. CC$_{50}$: 50% cytostatic concentration required to inhibit HEL cell proliferation by 50%.

Scheme 2. Proposed Metabolic Activation Route for Abacavir Phosphoramididates[a]



[a] (i) Esterase-type activity; (ii) spontaneous, probably via OPh displacement from the carboxylate (NGP); (iii) phosphoramidase. The ABCMP thus formed is processed via CBVMP to the bioactive CBVTP.

Table 5. Comparison of Succinate Salts of Separated Isomers to ~1:1 Mixtures of Diastereomers[a]

compd	HIV 1 EC$_{50}$ (μM) MT4	CC$_{50}$ (μM) MT4	HBV EC$_{50}$ (μM) HepG2 2.2.15	CC$_{50}$ (μM) HepG2 2.2.15
3a,**b** (mixture)	0.037	4.3	0.55	17
3a (fast)	0.027	1	0.4	10
3b (slow)	0.11	4	1.5	35
6a,**b** (mixture)	0.022	4.6	0.35	18
6a (fast)	0.0155	0.95	0.2	7.5
6b (slow)	0.14	5	0.27	35
1	2.15	89	5.6	110

[a] EC$_{50}$: 50% effective concentration required to inhibit HSV-induced cytopathicity by 50%. CC$_{50}$: 50% cytostatic concentration required to inhibit HEL cell proliferation by 50%.

ProTide, as much as 3020 pmol of CBVTP/10^9 CEM cells were found after 24 h of drug exposure, i.e., a 37-fold higher CBV triphosphate concentration than observed to appear in the presence of parental ABC. These markedly increased levels of active metabolite in the presence of ABC ProTide correspond well with a ~30-fold higher antiviral potency of the ProTide of ABC in CEM cell cultures than ABC. However, the highest metabolite levels were those afforded by the alaninyl ABC-MP derivative (**11**) together with CBV-MP, which were together 4-fold higher than those of CBV-TP. This is in sharp contrast with the alaninyl phosphoramidate d4T metabolite, which markedly accumulated (up to 100-fold) in aryloxymethylalaninyl d4TMP ProTide-exposed cell cultures compared with the d4TTP levels.[21] Thus, exposure of CEM cell cultures by the ProTide of ABC resulted in the eventual appearance of markedly higher intracellular CBV-TP levels, resulting in a more pronounced antiviral efficacy of the ProTide against HIV.

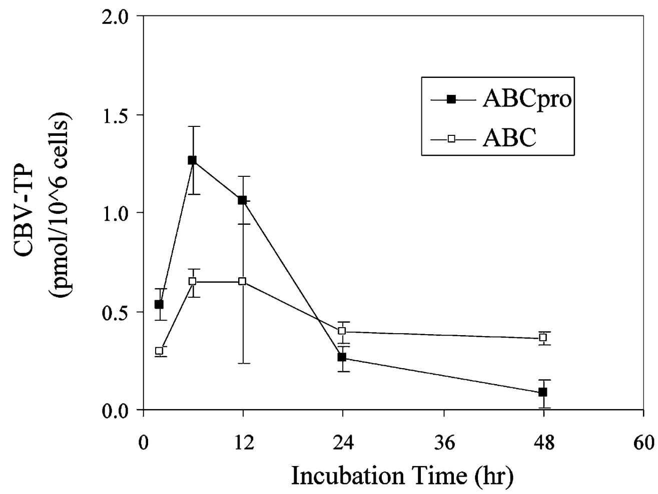


Figure 1. Intracellular levels of carbovir triphospate (CBV-TP) in HepG2 2.2.15 cells incubated with [8-^{3}H]abacavir (ABC, **1**) (10 μM, 2 mCi/μmol) or [8-^{3}H]abacavir 5′-[phenyl(methoxy-L-alaninyl)]phosphate (ABCpro, **3**) (1 μM, 2 mCi/μmol) for up to 48 h. Values are the mean ± standard deviation of triplicate determinations at each time point.

Metabolic profiles of [^{3}H]ABC and its ProTide **3** in HepG2 2.2.15 cells were qualitatively similar to those observed in CEM cells, with CBV-TP being the predominant radiolabeled anabolite. Peak CBV-TP levels in HepG2 2.2.15 cells incubated with [^{3}H]ABC (10 μM) or **3** (1 μM) were observed at 6−12 h (Figure 1).

Table 6. Metabolites of [^{3}H]ABC and the Aryloxymethylalaninyl ABC-MP ProTide of [^{3}H]ABC (**3**) in CEM Cell Cultures Exposed for 24 h to the Drugs

extracellular drug concn (10 μM)	metabolites of ABC and proABC (pmol/10^9 cells)						
	ABC + proABC + (CBV) fr 2−3	ABC + (CBV) fr 2−3	ABCMP fr 6−8	CBV-MP fr 11−13	CBVMP + alaninyl-ABCMP fr 11−13	CBVDP fr 19−21	CBVTP fr 35−38
1		762	5.8	108		42	82
3	5004		280		12410	3494	3020

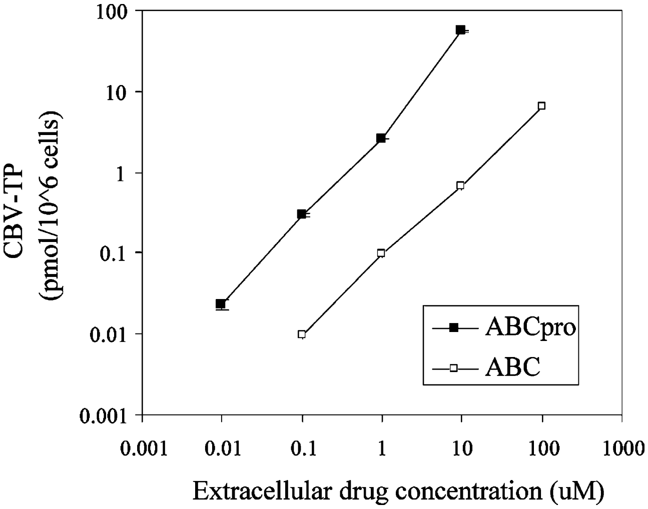


Figure 2. Intracellular levels of carbovir triphosphate (CBV-TP) in HepG2 2.2.15 cells incubated with various concentrations of [8-^{3}H]abacavir (ABC, **1**) (0.1, 1, 10, and 100 μM; 2 mCi/μmol) or [8-^{3}H]abacavir 5′-[phenyl(methoxy-L-alaninyl)]phosphate (ABCpro, **3**) (0.01, 0.1, 1, and 10 μM; 2 mCi/μmol) for 6 h. Values are the mean ± standard deviation of triplicate determinations at each concentration.

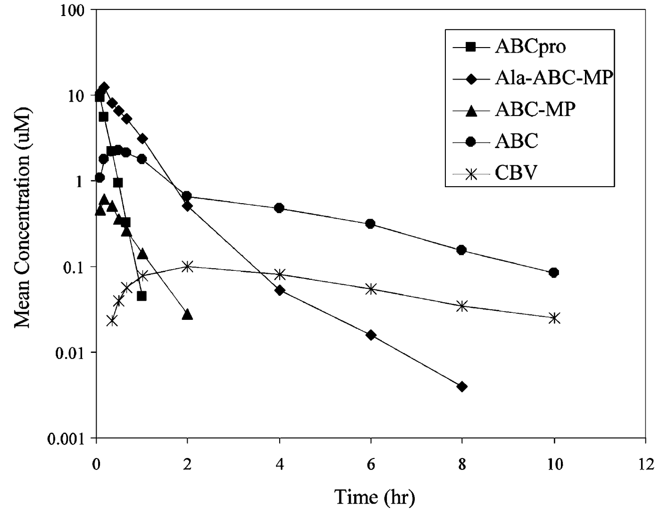


Figure 3. Plasma levels of abacavir 5′-[phenyl(ethoxy-L-alaninyl)]phosphate (ABCpro, **6**) and its metabolites, alaninyl ABC-MP (**11**), abacavir monophospate (ABC-MP), abacavir (ABC, **1**), and carbovir (CBV, **2**), in female cynomolgus monkeys following intravenous administration of ABCpro succinate salt (**6** succinate) at 11.5 mg/kg (17.3 μmol/kg).

Although the extracellular drug concentrations in this time course experiment were normalized relative to their potencies in the HBV assay (i.e., 5.6 or 0.55 μM, respectively; Table 2), the peak CBV-TP level produced by the ProTide (**3**) was approximately 2-fold higher than that produced by the parent compound (**1**). However, after 24- and 48-h incubations, CBV-TP levels in **3**-treated cells were below those in ABC-treated cells (Figure 1). Relative amounts of CBV-MP, -DP, and -TP produced by **3** and ABC at these time points were comparable, suggesting that the difference in anabolic efficiency over time may be attributable to a decrease in cellular uptake of **3** and/or its anabolism up to CBV-MP, but this could not be determined from these analyses because of coelution of **3**, ABC, and the upstream anabolites, i.e., alaninyl-ABC-MP and ABC-MP, in the chromatographic method used. In a separate dose response experiment, anabolism of ProTide to CBV-TP in HepG2 2.2.15 cells was shown to be quite efficient compared to that of ABC (Figure 2). Over a 100-fold drug concentration range (0.1−10 μM), peak CBV-TP levels provided by **3** were approximately 30- to 80-fold higher than those provided by an equivalent concentration of ABC. As seen in the time course experiment, the difference in peak CBV-TP levels produced by **3** versus ABC was greater than that predicted by its 10-fold improvement in anti-HBV activity (Table 2). This difference, along with the time-dependent CBV-TP profiles observed in the time course experiment (Figure 1), suggests that the kinetics of CBV-TP formation and/or clearance play a key role in determining in vitro potency and ultimately in vivo efficacy of such a ProTide.

Pharmacokinetics and Oral Bioavailability in Cynomolgus Monkeys. Following intravenous administration of its succinate salt (**6** succinate) (11.5 mg/kg or 17.3 μmol/kg) in cynomolgus monkeys, abacavir 5′-[phenyl(ethoxy-L-alaninyl)]phosphate (**6**) was rapidly cleared from plasma at a mean rate of 6.8 L h^{-1} kg^{-1} (~2.6-fold greater than hepatic blood flow in monkey[23]) with an elimination half-life of approximately 7 min (Table 7). Systemic exposure to ProTide (**6**) in the monkey was short-lived following either intravenous or oral administration, being at or below the limit of detection generally by 1−2 h postdose (Figures 3 and 4). Metabolism contributed significantly to the clearance

Table 7. Pharmacokinetic Parameter Value Estimates for Abacavir 5′-[Phenyl(ethoxy-L-alaninyl)]phosphate (ABCpro, **6**) and Its Metabolites, Alaninyl ABC-MP (**11**), Abacavir Monophospate (ABC-MP), Abacavir (ABC, **1**), and Carbovir (CBV, **2**), in Female Cynomolgus Monkeys Following Intravenous or Oral Administration of ABVpro Succinate Salt (**6** Succinate) at 11.5 mg/kg (17.3 μmol/kg)[a]

	pharmacokinetic parameter values[b]							
	intravenous			oral				
analyte	CL_p (L h^{-1} kg^{-1})	$t_{1/2}$ (hr)	AUC_{IV} (μM·h)	C_{max} (μM)	T_{max} (h)	$t_{1/2app}$ (h)	AUC_{PO} (μM·h)	F (%)
ABCpro	6.8 ± 2.0	0.11 ± 0.02	2.7 ± 0.8	0.6 ± 0.4	0.8 ± 0.3	0.4 ± 0.2[c]	0.7 ± 0.4[c]	22 ± 7[c]
Ala-ABC-MP	NA[d]	NA	9.2 ± 1.4	1.8 ± 1.1	1.3 ± 0.6	0.8 ± 0.1	2.6 ± 0.1	
ABC-MP	NA	NA	0.4 ± 0.1	0.1 ± 0.1	1.3 ± 0.6	NC[e]	NC	
ABC	NA	NA	6.0 ± 2.9	1.0 ± 0.8	3.0 ± 2.6	2.6 ± 1.0	6.0 ± 4.8	
CBV	NA	NA	0.7 ± 0.2	< 0.1	4.7 ± 1.2	6.0 ± 2.6	0.4 ± 0.1	
total			19.1 ± 1.5				9.5 ± 3.2	50 ± 15

[a] Values are the mean ± standard deviation for $n = 3$ except where noted. [b] CL_p, total plasma clearance of an intravenous dose; $t_{1/2}$, terminal elimination half-life following intravenous administration; $t_{1/2app}$, apparent elimination half-life following oral administration; AUC, area under the plasma concentration−time curve for an intravenous (iv) or oral (po) dose, extrapolated to infinity; C_{max}, maximum concentration observed after an oral dose; T_{max}, time of C_{max}; F, oral bioavailability. [c] Mean for $n = 2$. Limited data precluded calculation of these parameter values for one animal. [d] NA, not applicable. [e] NC, not calculable because of limited data for all three animals.

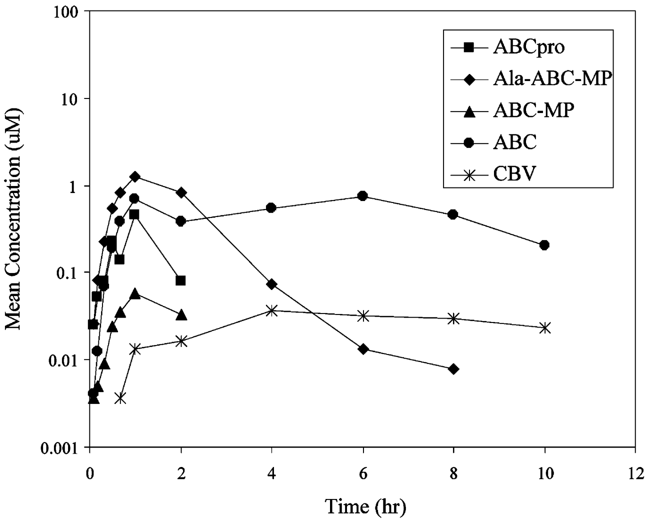


Figure 4. Plasma levels of abacavir 5′-[phenyl(ethoxy-L-alaninyl)]phosphate (ABCpro, **6**) and its metabolites, alaninyl ABC-MP (**11**), abacavir monophosphate (ABC-MP), abacavir (ABC, **1**), and carbovir (CBV, **2**), in female Cynomolgus monkeys following oral administration of ABCpro succinate salt (**6** succinate) at 11.5 mg/kg (17.3 μmol/kg).

of ProTide (**6**). This metabolism produced several active metabolites, albeit less potent than the ProTide itself (Table 4). Alaninyl-ABC-MP (**11**) was the predominant metabolite of intravenously administered ProTide, accounting for approximately 48% of the total area under the concentration−time curve (AUC), followed by ABC (**1**), which accounted for approximately 31% (Table 7). This order was reversed when the ProTide was administered orally, with ABC being the predominant metabolite and accounting for 63% of the total AUC (Table 7). ABC was the predominant active species in the plasma from 4 to 10 h after either dose (Figures 3 and 4). ABC-MP and CBV were also detected in plasma, but exposure to these metabolites was low, and all analytes were below detection at 24 h postdose. While exposure to the orally administered ProTide itself was low, total exposure to the ProTide and its active metabolites (9.5 $\pm$ 3.2 μM·h) (Table 7) approached that estimated for an equivalent dose of ABC (16 μM·h at 5 mg/kg or 17.5 μmol/kg ABC[24]). Perhaps even more noteworthy was the prolonged half-life of ABC derived from orally administered ProTide (2.6 $\pm$ 1.0 h) compared to orally administered parent (1.1 h [24]). These data are suggestive of a delayed release of ABC from the ProTide, with metabolism of the alaninyl-ABC-MP intermediate most likely being the rate-limiting step.

In conclusion, we herein report the application of the aryloxy phosphoramidate ProTide technology to the carbocyclic nucleoside analogues carbovir and abacavir. A significant (28- to 60-fold) increase in the in vitro anti-HIV potency of abacavir was noted on parent ProTide formation, with little enhancement in potency for carbovir. These data correlated well with observed enhancements in intracellular carbovir triphosphate levels from the abacavir ProTide. By contrast, in the anti-HBV assay, ProTide formation enhanced the potency of both abacavir and carbovir.

A small series of ester modified and amino acid modified analogues of the lead abacavir ProTide were prepared and studied, and initial SARs were established. It is notable that the unusual, achiral amino acid dimethylglycine emerged as a useful derivative, particularly in the anti-HBV assay.

Preliminary pharmacokinetic evaluation of one of the simpler abacavir ProTides, the phenylethoxyalaninyl phosphoramidate, in cynomolgus monkeys suggests that further optimization of these prodrugs for application to peripheral or systemic disease indications, e.g., HIV therapy, is required. Thus far, our attempts to enhance their metabolic stability, that is, their ability to survive first-pass (gut and liver) metabolism, while maintaining antiviral potency have indicated that this will be quite challenging within this class of compounds. However, sufficient delivery of intact ProTide to the liver may be achievable, but further studies are needed to explore this possibility.

Experimental Section

High-performance liquid chromatography (HPLC) was performed on an SSODS2 reverse-phase column with an eluent of water/acetonitrile. Method 1 involves the following parameters: 100% water (0 min), 20% water (35 min), 20% water (45 min), 100% water (55 min), with a flow rate of 1 mL/min and detection by UV at 254 nm. Standards are acetone (t_R = 4.54 min) and toluene (t_R = 10.21 min). Final product showed purities greater than 99%, with undetectable amounts of the parent nucleoside. For practical purposes, standard procedures are given where applicable.

Procedure A. Phenyl dichlorophosphate (1 mol equiv) and the appropriate amino acid ester hydrochloride salt (1 mol equiv) were suspended in anhydrous CH_2Cl_2 (30−60 mL). Anhydrous triethylamine (2 mol equiv) in CH_2Cl_2 (30 mL) was added dropwise at −80 °C, and the reaction was allowed to warm to room temperature overnight. The solvent was removed under reduced pressure and under nitrogen to give white solids. This solid was suspended in Et_2O (2 × 25 mL) and filtered, and the filtrate was concentrated in vacuo to give the products as crude oils. These were dissolved in anhydrous THF and used without further purification.

Procedure B. The nucleoside (1 mol equiv) was dried by azeotroping with anhydrous pyridine (3 × 5 mL) and then suspended in anhydrous THF (5−30 mL). To the suspension was added t-BuMgCl (1−2 mol equiv, 1.0 M solution in THF) dropwise, and the resulting suspension was stirred for 10 min. The phosphorochloridate (3 mol equiv, solution in THF) was then added dropwise, and the resulting solution was stirred at room temperature for 24−96 h. The reaction was quenched by the addition of saturated aqueous ammonium chloride (0.1 mL), and after 10 min the solvent was removed under reduced pressure. The crude product was purified by silica column chromatography.

Phenyl(methoxy-L-alaninyl) Phosphorochloridate (Methyl *N*-[Chloro(phenoxy)phosphoryl]-L-alaninate). This was synthesized according to procedure A, using L-alanine methyl ester hydrochloride (2.0 g, 14.34 mmol), phenyl phosphorodichloridate (3.02 g, 2.14 mL, 14.34 mmol), and anhydrous triethylamine (2.90 g, 4.0 mL, 28.68 mmol). The product (3.91 g, 98.2%) was isolated as a colorless crude oil. ^{1}H NMR ($CDCl_3$): δ 7.39−7.34 (2H, m), 7.29−7.20 (3H, m), 4.49−4.37 (1H, q), 4.27−4.09 (1H, m), 3.78 (3H, two s), 1.52−1.49 (3H, two d). ^{31}P NMR ($CDCl_3$): δ 9.28, 8.97 (1:1). ^{13}C NMR ($CDCl_3$): δ 173.6, 150.1, 130.25 (2C), 126.4, 120.9 (2C), 53.2, 51.0, 20.9.

(−)-Carbovir 5′-[Phenyl(methoxy-L-alaninyl)]phosphate (Methyl *N*-[{[(1*S*,4*R*)-4-(2-Amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)cyclopent-2-en-1-yl]methoxy}(phenoxy)phosphoryl]-L-alaninate) (10). (−)-Carbovir (300 mg, 1.21 mmol) was treated with t-BuMgCl (2.43 mL of a 1.0 M solution in THF, 2.43 mmol) and phenyl(methoxy-L-alaninyl) phosphorochloridate (5.32 mL of a 0.68 M solution in THF, 3.64 mmol) for 1 week according to procedure B to give after purification by column chromatography (3−5% MeOH in $CHCl_3$) the desired product as a white solid (136.5 mg, 23%).

^{1}H NMR($CDCl_3$): δ 7.67 (1H, two s), 7.37−7.30 (2H, m), 7.21−7.14 (3H, m), 6.17−6.10 (1H, m), 5.97−5.94 (1H, m), 5.53−5.48 (1H, m), 4.28−4.15 (2H, m), 4.00−3.87 (1H, m), 3.66 (3H, two s), 3.18 (1H, d), 2.83−2.71 (1H, m), 1.82−1.66 (1H, m), 1.36−1.29 (3H, two d). ^{31}P NMR (MeOH-d$_4$): δ 5.18, 4.86 (1:1). ^{13}C NMR ($CDCl_3$): δ 174.4, 158.5, 154.1, 151.7, 151.1, 136.9, 136.5, 130.7, 129.7 (2C), 125.0, 120.4 (2C), 116.8, 68.9, 59.8, 51.7, 50.5, 46.0, 34.2, 19.3. MS: *m/z* 489 (M + H). FAB: for $C_{21}H_{26}O_6N_6P$, requires 489.165 146, found 489.164 677. HPLC: t_R = 22.13, 22.51 (100%), method 1.

Abacavir 5′-[Phenyl(methoxy-L-alaninyl)]phosphate (Methyl *N*-[({(1*S*,4*R*)-4-[2-Amino-6-(cyclopropylamino)-9*H*-purin-9-yl]cyclopent-2-en-1-yl}methoxy)(phenoxy)-phosphoryl]-L-alaninate) (3a,b). Separation of Isomers. This was synthesized according to procedure B, using abacavir (500 mg, 1.75 mmol), t-BuMgCl (1.75 mL of 1.0 M solution in THF, 1.75 mmol), and phenyl(methoxy-L-alaninyl) phosphorochloridate (11.17 mL of 0.47 M solution in THF, 5.24 mmol) in THF (30 mL) and stirring at room temperature for 70 h. The crude product was purified by column chromatography (3% MeOH in CH_2Cl_2 and then 2% MeOH in CH_2Cl_2) to give **3** as a white foam after trituration with dichloromethane (442 mg, 48%). ^{1}H NMR ($CDCl_3$): δ 7.5 (1H, two s), 7.1−7.4 (5H, m), 6.1 (1H, m), 5.9 (2H, m), 5.5−5.6 (1H, m), 4.9 (2H, bs), 4.2 (2H, m), 4.05 (1H, m), 3.7 (3H, s), 3.6−3.8 (1H, m), 3.17 (1H, m), 3.0 (1H, m), 2.8 (1H, m), 1.7 (1H, m), 1.4 (3H, two d), 0.9 (2H, m), 0.6 (2H, m). ^{31}P NMR ($CDCl_3$): δ 3.07, 3.02. ^{31}P NMR (MeOH-*d*$_4$): δ 3.97, 3.88. ^{13}C NMR ($CDCl_3$): δ 174.6, 160.3, 156.6, 151.3, 151.1, 136.8, 135.9, 131.5, 130.0 (2C), 125.2, 120.5 (2C), 115.0, 69.2, 59.2, 52.8, 50.5, 46.0, 34.9, 24.2, 21.2, 7.7 (2C). MS: *m/z* 528 (M + H). MS FAB: $C_{24}H_{31}O_5N_7P$, requires 528.214 231, found 528.213 848. HPLC: t_R = 30.33 (100%), method 1. IR: 3328.6 (N−H str), 2922.1, 2862.9 (C−H str), 1734.4 (C=O str), 1590.9 (aromatic C−C str), 1462.9 (C−H def), 1376.8 (−CH_3 sym def), 1207.1 (P−O−aryl), 1154.0 (C−O str), 1027.7 (P−O−alkyl), 933.4 (olefinic C−H def), 721.8 (monosub aromatic C−H def). Anal. ($C_{24}H_{30}O_5N_7P$·0.4CH_2Cl_2) C, H, N.

The phosphate isomers were separated with supercritical fluid chromatography using a Chiralpak AS column and 25% methanol in carbon dioxide as the eluent. The first isomer to elute with a t_R of 2.9 min from a Chiralpak AS column (25% methanol in carbon dioxide, flow rate of 2 mL/min, temperature of 40 °C, 3000 psi, enantiopure) and upon evaporation of solvents gave the isomer **3a** as a white foam after trituration with chloroform (recovery of >95%). ^{1}H NMR ($CDCl_3$): δ 7.50 (1H, s), 7.3−7.4 (2H, m), 7.15−7.25 (3H, m), 6.11 (1H, m), 5.91 (1H, m), 5.86 (1H, s), 5.55 (1H, m), 4.89 (2H, s), 4.24 (2H, m), 4.05 (1H, m), 3.72 (3H, s), 3.65 (1H, m), 3.20 (1H, m), 3.02 (1H, m), 2.83 (1H, m), 1.72 (1H, m), 1.37 (3H, d), 0.89 (2H, m), 0.62 (2H, m). ^{31}P NMR ($CDCl_3$): δ 3.07. Anal. ($C_{24}H_{30}N_7O_5P$·0.14$CHCl_3$) C, H, N.

The second isomer to elute with a t_R of 6.7 min from a Chiralpak AS column (25% methanol in carbon dioxide, flow rate of 2 mL/min, temperature of 40 °C, 3000 psi, enantiopure) and upon evaporation of solvents gave the isomer **3b** as a white foam after trituration with chloroform. ^{1}H NMR ($CDCl_3$): δ 7.52 (1H, s), 7.25−7.4 (2H, m), 7.15−7.22 (3H, m), 6.11 (1H, m), 5.94 (1H, m), 5.85 (1H, s), 5.55 (1H, m), 4.88 (2H, s), 4.22 (2H, m), 4.04 (1H, m), 3.75 (3H, s), 3.7−3.75 (1H, m), 3.17 (1H, m), 3.04 (1H, m), 2.80 (1H, m), 1.73 (1H, m), 1.42 (3H, d), 0.89 (2H, m), 0.67 (2H, m). ^{31}P NMR ($CDCl_3$): δ 3.0. Anal. ($C_{24}H_{30}N_7O_5P$·0.2$CHCl_3$) C, H, N.

Abacavir 5′-[Phenyl(methoxy-L-alaninyl)]phosphate Succinate Salt (3 Succinate). Abacavir 5′-[phenyl(methoxy-L-alaninyl)]phosphate (1:1 mixture of isomers of **3a**,**b**, 100 mg, 0.19 mmol) was dissolved in methanol. To this solution was added succinic acid (22 mg, 0.19 mmol), and the resulting solution was evaporated to dryness. The residue was dissolved in acetonitrile (10 mL) with heating. Precipitate formed upon cooling. The mixture was stored in the refrigerator overnight and the solid was collected by filtration to give 70 mg (57%) of a 1:1 mixture of **3** succinate as a crystalline solid. ^{1}H NMR (DMSO-*d*$_6$): δ 12.15 (2H, s, D_2O exchangeable), 7.61 (1H, s), 7.36 (3H, becomes 2H on D_2O exchange), 7.20 (3H, m), 5.9−6.1 (3H, m), 5.88 (2H, bs, D_2O exchangeable), 5.44 (1H, m), 4.0−4.2 (2H, m), 3.85 (1H, m), 3.60 (3H, s), 3.05 (2H, m), 2.65 (1H, m), 2.44 (4H, s), 1.64 (1H, m), 1.23 (3H, m), 0.5−0.7 (4H, m). ^{31}P NMR (DMSO-*d*$_6$): δ 3.99 and 3.66. Anal. ($C_{24}H_{30}N_7O_5P$·$C_4H_6O_4$·0.5H_2O) C, H, N.

Phenyl(methoxy-D-alaninyl)phosphorochloridate (Methyl *N*-[Chloro(phenoxy)phosphoryl]-D-alaninate). This was synthesized according to procedure A, using D-alanine methyl ester hydrochloride (1.0 g, 7.17 mol), phenyl phosphorodichloridate (1.51 g, 1.07 mL, 7.17 mmol), and anhydrous triethylamine (1.45 g, 2.0 mL, 14.0 mmol) to yield 1.66 g (83.4%) of product. ^{1}H NMR ($CDCl_3$): δ 7.39−7.30 (2H, t), 7.29−7.09 (3H, m), 4.85−4.80 (1H, d), 4.19−4.11 (1H, m), 3.75 (3H, two s), 1.52−1.49 (3H, two d). ^{31}P NMR ($CDCl_3$): δ 9.38, 9.18 (1:1). ^{13}C NMR ($CDCl_3$): δ 173.6, 150.1, 130.3 (2C), 126.4 (2C), 120.9, 53.2, 50.9, 21.0.

Abacavir 5′-[Phenyl(methoxy-D-alaninyl)]phosphate (Methyl *N*-[({(1*S*,4*R*)-4-[2-Amino-6-(cyclopropylamino)-9*H*-purin-9-yl]cyclopent-2-en-1-yl}methoxy)(phenoxy)-phosphoryl]-D-alaninate) (4). This was synthesized according to procedure B, using abacavir (400 mg, 1.4 mmol), t-BuMgCl (2.1 mL of 1.0 M solution in THF, 2.1 mmol), and phenyl(methoxy-D-alaninyl) phosphorochloridate (7.0 mL of 0.6 M solution in THF, 4.19 mmol) in THF (25 mL) stirring at room temperature for 36 h. The crude product was purified by column chromatography (3% MeOH in $CHCl_3$ and then 2.5% MeOH in $CHCl_3$) to give **4** as a white foam (318.6 mg, 43.2%). ^{1}H NMR ($CDCl_3$): δ 7.53 (1H, two s), 7.37−7.32 (2H, m), 7.29 (1H, d), 7.25−7.15 (2H, m), 6.10 (1H, t, J = 5.28 Hz), 6.03 (1H, bs, NHcPr), 5.94−5.89 (1H, m), 5.54 (1H, m), 5.01 (2H, bs, NH_2), 4.26−3.83 (4H, m), 3.72 (3H, two s), 3.18 (1H, s), 3.02 (1H, bs), 2.86−2.75 (1H, m), 1.78−1.64 (1H, m), 1.39−1.36 (3H, two d), 0.90−0.83 (2H, q, J = 6.13 Hz), 0.63 (2H, bs). ^{31}P NMR ($CDCl_3$): δ 3.93, 3.70. ^{13}C NMR ($CDCl_3$): δ 174.5, 160.3, 156.6, 151.2, 151.0, 136.8, 136.1, 131.5, 130.0 (2C), 125.3, 120.5 (2C), 115.2, 69.3, 59.3, 52.9, 50.5, 46.0, 34.9, 24.1, 21.4, 7.8 (2C). MS: *m/z* 528 (M + H). MS FAB: for $C_{24}H_{31}O_5N_7P$ requires 528.212 431, found 528.211 505. HPLC: t_R = 29.807 (100%), method 1. IR: 3333.6 (N−H str), 2923.4, 2853.4 (C−H str), 1734.1 (C=O str), 1591.1 (aromatic C−C str), 1458.3 (C−H def), 1376.7 (−CH_3 sym def), 1208.3 (P−O−aryl), 1153.3 (C−O str), 1026.9 (P−O−alkyl), 931.9 (olefinic C−H def), 721.6 (monosub aromatic C−H def). Anal. ($C_{24}H_{30}O_5N_7P$) C, H, N.

Phenyl(methoxyglycinyl) Phosphorochloridate (Methyl *N*-[Chloro(phenoxy)phosphoryl]glycinate). This was synthesized according to procedure A, using glycine methyl ester (1.5 g, 11.9 mmol), phenyl phosphorodichloridate (2.52 g, 1.79 mL, 11.9 mmol), and anhydrous triethylamine (2.42 g, 3.33 mL, 23.9 mmol) to yield 3.07 g (97.15%) of the product as an oil. ^{1}H NMR ($CDCl_3$): δ 7.43−7.38 (2H, m), 7.31−7.25 (3H, m), 4.67 (1H, bs, NHala), 3.94 (2H, dd), 3.83 (3H, s). ^{31}P NMR ($CDCl_3$): δ 10.43. ^{13}C NMR ($CDCl_3$): δ 170.4, 150.1, 130.2 (2C), 126.4, 120.8 (2C), 53.1, 43.4.

Abacavir 5′-[Phenyl(methoxyglycinyl)]phosphate (Methyl *N*-[({(1*S*,4*R*)-4-[2-Amino-6-(cyclopropylamino)-9*H*-purin-9-yl]cyclopent-2-en-1-yl}methoxy)(phenoxy)-phosphoryl]glycinate) (5). This was synthesized according to procedure B, using abacavir (300 mg, 1.05 mmol), t-BuMgCl (1.57 mL of 1.0 M solution in THF, 1.57 mmol), and phenyl-(methoxyglycinyl) phosphorochloridate (4.06 mL of 0.774 M solution in THF, 3.14 mmol) in THF (20 mL) stirring at room temperature for 96 h. The crude product was purified by column chromatography (3% MeOH in $CHCl_3$ and then with 2.5% MeOH in $CHCl_3$) to give **5** as a white foam (82.6 mg, 15.4%). ^{1}H NMR ($CDCl_3$): δ 7.38 (1H, two s), 7.24−7.19 (2H, t), 7.15−7.10 (2H, t), 7.07−7.02 (1H, t), 6.00−5.96 (2H, m), 5.80−5.76 (1H, m), 5.45−5.41 (1H, t), 4.99 (2H, bs, NH_2), 4.14−4.00 (3H, m), 3.62 (3H, s), 3.03 (1H, d), 2.91 (1H, d), 2.73−2.62 (1H, m), 1.62−1.51 (1H, m), 1.45−1.43 (2H, m), 0.78−0.71 (2H, q), 0.54−0.49 (2H, t). ^{31}P NMR ($CDCl_3$): δ 4.79, 4.67 (1:1). ^{13}C NMR($CDCl_3$): δ 172.1, 160.2, 156.6, 152.0, 151.7, 137.7, 137.1, 132.0, 130.8 (2C), 126.0, 121.2 (2C), 115.5, 69.9, 60.0, 53.5, 46.7, 43.9, 35.4, 25.0, 8.5 (2C). MS: *m/z* 514 (M +

H). MS FAB: $C_{23}H_{29}O_5N_7P$ requires 514.196 781, found 514.195 321. HPLC: t_R = 28.419 (99.9%), method 1. IR: 3342.0 (N−H str), 1749.8 (C═O str), 1596.2, 1488.4 (aromatic C−C str), 1451.9 (C−H def), 1394.7 (−CH_3 sym def), 1259.6 (P═O), 1212.1 (P−O-aryl), 1151.6 (C−O str), 1026.8 (P−O−alkyl), 937.8 (olefinic C−H def), 760.7 (monosub aromatic C−H def).

Phenyl(ethoxy-L-alaninyl) Phosphorochloridate (Ethyl *N*-[Chloro(phenoxy)phosphoryl]-L-alaninate). This was synthesized according to procedure A, using L-alanine ethyl ester hydrochloride (1.0 g, 6.51 mmol), phenyl phosphorodichloridate (1.37 g, 0.97 mL, 6.51 mmol), and anhydrous triethylamine (1.32 g, 1.81 mL, 13.0 mmol) to yield 1.85 g (97.4%) of the desired product as an oil. ^{1}H NMR($CDCl_3$): δ 7.42−7.35 (2H, dd), 7.31−7.25 (3H, m), 4.71 (1H, d, NHala), 4.31−4.13 (3H, m), 1.55−1.52 (3H, m), 1.33−1.30 (3H, two d). ^{31}P NMR ($CDCl_3$): δ 9.41, 9.16 (1:1). ^{13}C NMR: δ 173.1, 150.2, 130.3 (2C), 126.4, 120.9 (2C), 62.3, 51.0, 20.9, 14.5.

Abacavir 5′-[Phenyl(ethoxy-L-alaninyl)]phosphate (Ethyl *N*-[({(1*S*,4*R*)-4-[2-Amino-6-(cyclopropylamino)-9*H*-purin-9-yl]cyclopent-2-en-1-yl}methoxy)(phenoxy)phosphoryl]-L-alaninate) (6). This was synthesized according to procedure B, using abacavir (300 mg, 1.4 mmol), t-BuMgCl (1.57 mL of 1.0 M solution in THF, 1.57 mmol), and phenyl-(ethoxy-L-alaninyl) phosphorochloridate (6.45 mL of 0.49 M solution in THF, 3.14 mmol) in anhydrous THF (20 mL) and stirring at room temperature for 24 h. The crude product was purified by column chromatography (2.5% MeOH in $CHCl_3$) to give **6** as a pale-yellow foam (290 mg, 51.1%). ^{1}H NMR ($CDCl_3$): δ 7.47 (1H, two s), 7.10−7.46 (5H, m), 6.07 (1H, m), 5.9 (1H, m), 5.78 (1H, s), 5.5 (1H, m), 4.84 (2H, bs), 4.1 (4H, m), 4.00 (1H, m), 3.64 (1H, m), 3.14 (1H, m), 3.0 (1H, m), 2.78 (1H, m), 1.68 (1H, m), 1.36 (3H, two d), 1.22 (3H, two t), 0.86 (2H, m), 0.6 (2H, m). ^{31}P NMR ($CDCl_3$): δ 3.05, 3.02. ^{31}P NMR: δ 4.04, 3.96 (1:1). ^{13}C NMR: δ 173.35, 159.8, 156.0, 150.6, 150.4, 136.1, 135.1, 130.8, 129.3 (2C), 124.5, 119.8 (2C), 114.4, 68.6, 61.2, 58.5, 50.0, 45.3, 34.3, 23.4, 20.6, 13.8, 7.0 (2C). MS: *m/z* 542 (M + H). MS FAB: $C_{25}H_{33}O_5N_7P$, requires 542.228 081, found 542.228 131. HPLC: t_R = 31.76, 32.03 (100%), method 1. IR: 3334.1 (N−H str), 1734.5 (C═O str), 1595.9, 1488.0 (aromatic C−C str), 1450.3 (C−H def), 1394.2 (−CH_3 sym def), 1252.8 (P═O), 1210.4 (P−O-aryl), 1153.3 (C−O str), 1026.0 (P−O-alkyl), 934.8 (olefinic C−H def), 759.0 (monosub aromatic C−H def). Anal. ($C_{25}H_{32}O_5N_7P$·0.25CH_2Cl_2) C, H, N.

Abacavir 5′-[Phenyl(ethoxy-L-alaninyl)]phosphate Succinate Salt (6 Succinate). Abacavir 5′-[phenyl(ethoxy-L-alaninyl)]phosphate (1:1 mixture of isomers of **6**, 376 mg, 0.7 mmol) was dissolved in ethanol. To this solution was added succinic acid (82 mg, 0.7 mmol), and the resulting solution was evaporated to dryness. The residue was dissolved in acetonitrile (10−20 mL) with heating. Precipitate formed upon cooling. The mixture was stored in the refrigerator overnight and the solid was collected by filtration to give 330 mg (72%) of a 1:1 mixture of **6** succinate as a crystalline solid. ^{1}H NMR (DMSO-d_6): δ 12.14 (2H, s), 7.58 (1H, s), 7.1−7.4 (6H, m), 5.9−6.1 (3H, m), 5.85 (2H, bs), 5.42 (1H, m), 3.95−4.15 (4H, m), 3.8 (1H, m), 3.05 (2H, m), 2.65 (1H, m), 2.4 (4H, s), 1.63 (1H, m), 1.4 (3H, two d), 1.12 (3H, t), 0.5−0.7 (4H, m). ^{31}P NMR (DMSO-d_6): δ 4.00 and 3.68. FAB MS: calcd for $C_{25}H_{32}N_7O_5P$ $(M + H)^+$ (*m/z*) 542.2281, found 542.2282. Anal. ($C_{25}H_{32}N_7O_5P$·$C_4H_6O_4$·0.5H_2O) C, H, N.

Abacavir 5′-[Phenyl(ethoxy-L-alaninyl)]phosphate Fumarate Salt (6 Fumarate). Abacavir 5′-[phenyl(ethoxy-L-alaninyl)]phosphate (1:1 mixture of isomers of **6**, 198 mg, 0.37 mmol) was dissolved in ethanol. To this solution was added fumaric acid (43 mg, 0.37 mmol), and the resulting solution was evaporated to dryness. The residue was dissolved in acetonitrile (10 mL) with heating. Precipitate formed upon cooling. The mixture was stored in the refrigerator overnight and the solid was collected by filtration to give 185 mg (75%) of a 1:1 mixture of **6** fumarate as a crystalline solid. ^{1}H NMR (DMSO-d_6): δ 7.6 (1H, s), 7.1−7.4 (6H, m), 6.64 (2H, s), 5.9−6.1 (3H, m), 5.87 (2H, bs), 5.44 (1H, m), 3.95−4.15 (4H, m), 3.84 (1H, m), 3.05 (2H, m), 2.65 (1H, m), 1.63 (1H, m), 1.23 (3H, m), 1.15 (3H, t), 0.5−0.7 (4H, m). ^{31}P NMR (DMSO-d_6): δ 4.00 and 3.67. Anal. ($C_{25}H_{32}N_7O_5P$·$C_4H_4O_4$·0.5H_2O) C, H, N.

Abacavir 5′-[Phenyl(ethoxy-L-alaninyl)]phosphate Glutarate Salt (6 Glutarate). Abacavir 5′-[phenyl(ethoxy-L-alaninyl)]phosphate (1:1 mixture of isomers of **6**, 200 mg, 0.38 mmol) was dissolved in ethanol. To this solution was added glutaric acid (50 mg, 0.38 mmol), and the resulting solution was evaporated to dryness. The residue was dissolved in acetonitrile (10 mL) with heating. The mixture was stored in the refrigerator overnight and the solid was collected by filtration to give 130 mg (50%) of a 1:1 mixture of **6** glutarate as a crystalline solid. ^{1}H NMR (DMSO-d_6): δ 7.6 (1H, s), 7.1−7.4 (6H, m), 5.9−6.1 (3H, m), 5.87 (2H, bs), 5.44 (1H, m), 3.95−4.2 (4H, m), 3.8 (1H, m), 3.1 (m, 2H), 2.65 (1H, m), 2.25 (4H, t), 1.7 (3H, m), 1.23 (3H, m), 1.15 (3H, t), 0.5−0.7 (4H, m). ^{31}P NMR (DMSO-d_6): δ 4.00 and 3.68. Anal. ($C_{25}H_{32}N_7O_5P$·$C_5H_8O_4$·0.5H_2O) C, H, N.

Abacavir 5′-[Phenyl(ethoxy-L-alaninyl)]phosphate Succinate Salt (6 Succinate). Separation of Isomers and Preparation of Succinates (6a,b Succinate). For scaling up of abacavir 5′-[phenyl(ethoxy-L-alaninyl)]phosphate succinate salt a modification of procedure B was used. Thus, to abacavir (15 g, 52 mmol) was added anhydrous pyridine (200 mL). Subsequently, t-BuMgCl (60 mL, 1 M solution in THF, 60 mmol) was added slowly. The reaction mixture was stirred for 20 min, and a solution of phenylethoxy-L-alaninyl phosphorochloridate (31 g, 107 mmol in 100 mL of THF, synthesized according to procedure A) was added. The reaction mixture was stirred at room temperature for 10 h and subsequently concentrated in vacuo to a brown syrup. This syrup was dissolved in CH_2Cl_2 (100 mL), and the CH_2Cl_2 was extracted with water (2 × 100 mL) and saturated aqueous sodium bicarbonate, dried ($MgSO_4$), filtered, and concentrated to a brown foam. This solid foam was purified by filtration through a silica gel plug (100 g, silica, 5% methanol in chloroform) to give 18.5 g (67%) of **6** as a white foam. This foam (**6**, 18.5 g, 34 mmol) was dissolved in ethanol (150 mL). To this was added succinic acid (4.0 g, 34 mmol), and the resulting solution was stirred for 30 min, then concentrated to dryness. The resulting solid was dissolved in refluxing acetonitrile (100 mL), then allowed to crystallize upon cooling to give 18.8 g (84%) of **6** succinate as a crystalline solid.

The phosphate isomers of **6** were separated with supercritical fluid chromatography using a Chiralpak AS column and 22% methanol in carbon dioxide as the eluent. The first isomer to elute with a t_R of 3.2 min from a Chiralpak AS column (25% methanol in carbon dioxide, flow rate of 2 mL/min, temperature of 40 °C, 3000 psi, enantiopure) and upon evaporation of solvents gave the isomer (**6a**) as a white foam (recovery of >95%). Treatment of this foam with succinic acid as described above gave the enantiopure faster-eluting isomer as a crystalline succinate salt (**6a** succinate). ^{1}H NMR (DMSO-d_6): δ 12.14 (2H, s), 7.57 (1H, s), 7.1−7.4 (6H, m), 5.9−6.1 (3H, m), 5.85 (2H, bs), 5.42 (1H, m), 3.95−4.15 (4H, m), 3.8 (1H, m), 3.05 (2H, m), 2.65 (1H, m), 2.4 (4H, s), 1.63 (1H, m), 1.4 (3H, two d), 1.12 (3H, t), 0.5−0.7 (4H, m). ^{31}P NMR (DMSO-d_6): δ 4.00. Anal. ($C_{25}H_{32}N_7O_5P$·$C_4H_6O_4$·0.5H_2O) C, H, N.

The second isomer to elute with a t_R of 7.4 min from a Chiralpak AS column (22% methanol in carbon dioxide, flow rate of 2 mL/min, temperature of 40 °C, 3000 psi, enantiopure) and upon evaporation of solvents gave the isomer **6b** as a white foam. Treatment of this foam with succinic acid as described above gave the enantiopure slower-eluting isomer as a crystalline succinate salt (**6b** succinate). ^{1}H NMR (DMSO-d_6): δ 12.14 (2H, s), 7.58 (1H, s), 7.1−7.4 (6H, m), 5.9−6.1 (3H, m), 5.85 (2H, bs), 5.42 (1H, m), 3.95−4.15 (4H, m), 3.8 (1H, m), 3.05 (2H, m), 2.65 (1H, m), 2.4 (4H, s), 1.63 (1H, m), 1.4 (3H, two d), 1.12 (3H, t), 0.5−0.7 (4H, m). ^{31}P NMR (DMSO-d_6): δ 3.68. Anal. ($C_{25}H_{32}N_7O_5P$·$C_4H_6O_4$) C, H, N.

Phenyl(isopropoxy-L-alaninyl) Phosphorochloridate (Isopropyl *N*-[Chloro(phenoxy)phosphoryl]-L-alaninate). This was synthesized according to procedure A, using L-alanine isopropyl ester hydrochloride salt (0.5 g, 2.98 mmol), phenyl phosphorodichloridate (0.45 mL, 2.98 mmol), and triethylamine (0.83 mL 5.97 mmol) in CH_2Cl_2 (70 mL) to yield the product (1.12 g, quant,) as a yellow oil. ^{31}P NMR ($CDCl_3$): δ 9.45, 9.17 (1:1). ^{13}C NMR ($CDCl_3$): δ 172.6, 150.2, 130.3 (2C), 126.4, 121.0 (2C), 70.1, 51.1, 22.1, 20.9.

Abacavir 5′-Phenyl(isopropoxy-L-alaninyl)phosphate (Isopropyl *N*-[({(1*S*,4*R*)-4-[2-Amino-6-(cyclopropylamino)-9*H*-purin-9-yl]cyclopent-2-en-1-yl}methoxy)(phenoxy)-phosphoryl]-L-alaninate) (7). This was synthesized according to procedure A, using abacavir (100 mg, 0.35 mmol), t-BuMgCl (0.7 mL of a 1.0 M solution in THF, 0.7 mmol,) in THF (3 mL), and phenyl(isopropyl-L-alaninyl) phosphorochloridate (1.76 mL of a 0.597 M solution in THF, 1.05 mmol) at room temperature for 72 h. The crude product was purified by column chromatography (3% MeOH in $CHCl_3$) to give **7** (106.8 mg, 54.8%) as a pale-yellow foamy solid. ^1H NMR ($CDCl_3$): δ 7.41 (1H, two s), 7.24−7.19 (2H, m), 7.13−7.03 (3H, m), 6.37 (1H, bs, NHcPr), 5.98 (1H, t), 5.80−5.76 (1H, m), 5.43 (1H, bs), 5.21 (2H, bs, NH_2), 4.94−4.86 (1H, m), 4.15−3.98 (2H, m), 3.92−3.83 (1H, m), 3.59 (1H, bs, NHala), 3.06−2.98 (1H, m), 2.93 (1H, bs), 2.74−2.63 (1H, m), 1.62−1.53 (1H, m), 1.34−1.18 (3H, m), 1.15−1.11 (6H, m), 0.79−0.73 (2H, q), 0.53 (2H, m). ^{31}P NMR ($CDCl_3$): δ 4.02, 3.98 (1:1). ^{13}C NMR: δ 173.5, 159.8, 156.2, 151.1, 151.0, 136.9, 136.1, 131.3, 130.0 (2C), 125.3, 120.5 (2C), 115.0, 69.6, 69.2, 59.3, 50.7, 46.0, 34.9, 24.2, 22.0 (2C), 21.4, 7.8 (2C). MS: *m/z* 557 (M + H). MS MALD/I TOF: found 555.6, $C_{26}H_{34}O_5N_7P$ requires 555.2. HPLC: t_R = 35.85 (100%), method 1.

Phenyl-*tert*-butyloxy-L-alaninylphosphorochloridate (*tert*-Butyl *N*-[Chloro(phenoxy)phosphoryl]-L-alaninate). This was synthesized according to procedure A, using L-alanine *tert*-butyl ester hydrochloride (0.5 g, 2.75 mmol), phenyl phosphorodichloridate (0.41 mL, 2.75 mmol), and triethylamine (0.77 mL, 5.5 mmol) to yield 0.77 g (87.5%) of product. ^1H NMR($CDCl_3$): δ 7.44−7.39 (2H, t), 7.32−7.26 (3H, m), 4.47−4.34 (1H, m, NHala), 4.17−4.04 (1H, m), 1.53 (9H, s). ^{31}P NMR ($CDCl_3$): δ 9.53, 9.20 (1:1). ^{13}C NMR ($CDCl_3$): δ 170.7, 148.7, 128.9 (2C), 124.9, 119.5 (2C), 81.65, 50.0, 26.9 (3C).

Abacavir 5′-(Phenyl-*tert*-butyloxy-L-alaninyl)phosphate (*tert*-Butyl *N*-[({(1*S*,4*R*)-4-[2-Amino-6-(cyclopropylamino)-9*H*-purin-9-yl]cyclopent-2-en-1-yl}methoxy)(phenoxy)-phosphoryl]-L-alaninate) (8). This was synthesized according to procedure B, using abacavir (140 mg, 0.52 mmol), t-BuMgCl (1.05 mL of a 1.0 M solution in THF, 1.05 mmol), and phenyl(*tert*-butyloxy-L-alaninyl) phosphorochloridate (3.3 mL of a 0.48 M solution in THF, 1.57 mmol) in anhydrous THF (4 mL) stirring at room temperature for 48 h. The crude product was purified by column chromatography (3% MeOH in $CHCl_3$) to give **8** (192.3 mg, 69.0%) as a white foam. ^1H NMR ($CDCl_3$): δ 7.40 (1H, two s), 7.23−7.18 (2H, t), 7.12 (2H, d), 7.06−7.02 (1H, t), 6.09 (1H, m), 5.97 (1H, m), 5.77 (1H, d, NHcPr), 5.44 (1H, m), 5.10 (2H, bs, NH_2), 4.14−4.05 (3H, m), 3.85−3.77 (1H, q), 3.04 (1H, bs), 2.93 (1H, m), 2.72−2.62 (1H, m), 1.58−1.53 (1H, t), 1.34 (9H, two s), 1.27−1.23 (3H, t), 0.73 (2H, d), 0.51 (2H, m). ^{31}P NMR ($CDCl_3$): δ 4.15 (s). ^{13}C NMR ($CDCl_3$): δ 173.2, 160.4, 156.7, 151.2 (2C), 136.8, 135.9, 131.5, 130.0 (2C), 125.2, 120.6 (2C), 115.2, 82.3, 69.3, 59.1, 46.0, 35.0, 28.3 (3C), 24.2, 21.5, 7.8 (2C). MS: *m/z* 571 (M + H). MS FAB: For $C_{27}H_{37}O_5N_7P$ requires 570.2594, found 570.2598. HPLC: t_R = 36.158 (100%), method 1. Anal. ($C_{27}H_{36}O_5N_7P{\cdot}0.33H_2O$) C, H, N.

Abacavir 5′-(Phenylmethoxydimethylglycinyl)-phosphate (*tert*-Butyl *N*-[({(1*S*,4*R*)-4-[2-Amino-6-(cyclopropylamino)-9*H*-purin-9-yl]cyclopent-2-en-1-yl}-methoxy)(phenoxy)phosphoryl]dimethylglycinate) (9). This was synthesized according to standard procedure 4, using abacavir (300 mg, 1.05 mmol), t-BuMgCl (1.0M solution in THF) (1.57 mL, 1.57 mmol), and phenyl(methoxydimethylglycinyl) phosphorochloridate (0.59 M solution in THF) (5.3 mL, 3.14 mmol) in THF (20 mL) stirring at room temperature for 96 h. The crude product was purified by eluting with 3% MeOH in $CHCl_3$ and then with 2.5% MeOH in $CHCl_3$ to give the product **9** as a white foam (193.7 mg, 34.14%). ^1H NMR-($CDCl_3$): δ 7.40 + 7.36 (1H, 2 × S), 7.24 − 7.19 (2H, t), 7.15−7.10 (2H, t), 7.07−7.02 (1H, t), 6.00−5.96 (2H, m), 5.80−5.76 (1H), 5.45−5.41 (1H, t), 4.99 (2H, bs, NH_2), 4.14−4.00 (3H, m), 3.62 (3H, s), 3.03 (1H, d), 2.91 (1H, d), 2.73−2.62 (1H, m), 1.62−1.51 (1H, m, 1 of H6′), 1.45−1.43 (6H, t, 2 × CH_3), 0.78−0.71 (2H, q, 2H of CH_2cPr), 0.54−0.49 (2H). ^{31}P NMR ($CDCl_3$): δ 2.49. MS ES$^+$: *m/z* 541.9 (100%, M$^+$), 563.8 (30%, M + Na$^+$). MS FAB: for $C_{25}H_{33}O_5N_7P$ requires 542.228 081, found 542.228 428. HPLC: t_R = 28.347 (100%), method 1. IR: 3346.0 (N−H str), 2923.0, 2853.5 (C−H str), 1734.0 (C═O str), 1590.2 (aromatic C−C str), 1458.4 (C−H def), 1376.8 (−CH_3 sym def), 1261.3 (P═O), 1152.7 (C−O str), 1028.0 (P−O-alkyl), 936.0 (olefinic C−H def), 721.7 (monosub aromatic C−H def).

Abacavir 5′-(L-Alaninyl)phosphate Disodium Salt. (*N*-[({(1*S*,4*R*)-4-[2-Amino-6-(cyclopropylamino)-9*H*-purin-9-yl]cyclopent-2-en-1-yl}methoxy)phosphoryl]-D-alaninate Disodium Salt) (11). Abacavir 5′-[phenyl(methoxy-L-alaninyl)]phosphate (1:1 mixture of isomers of **3a** and **3b**, 500 mg, 0.95 mmol) was suspended in a solution of triethylamine (30 mL) and deionized water (30 mL) and stirred at room temperature for 18 h. The resulting solution was evaporated to dryness in vacuo, and the residue was dissolved in water (50 mL), extracted with dichloromethane (2 × 50 mL), and purified by anion exchange chromatography using a Sep-Pak Vac 35 cm^3 Accell Plus QMA cartridge (Waters Corp., P/N WAT054725) (HCO_3^- form) with an aqueous ammonium bicarbonate buffer (0−0.5 M linear gradient, 1 L). The appropriate fractions were combined and evaporated to dryness in vacuo. The residue was twice dissolved in deionized water and evaporated to dryness in vacuo to give **11** as the ammonium salt. This salt was dissolved in deionized water and passed through a Sep-Pak Vac 20 cm^3 Accell Plus CM cartridge (Waters Corp., P/N WAT054675) (Na^+ form) using deionized water. The appropriate fractions were combined and lyophilized to give 0.430 g (86% yield) of **11** disodium salt 2.5 hydrate as a white solid. ^1H NMR (D_2O): δ 7.71 (1H, s), 6.11 (1H, m), 5.80 (1H, m), 5.32 (1H, m), 3.66 (2H, m), 3.41 (1H, m), 2.99 (1H, m), 2.68 (2H, m), 1.49 (1H, m), 1.12 (3H, d), 0.74 (2H, m), 0.54 (2H, m). ^{31}P NMR (D_2O): δ 8.68. MS (ES$^-$) *m/z* 436 (M − H). Anal. ($C_{17}H_{22}N_7Na_2O_5P{\cdot}2.5H_2O$) C, H, N.

Abacavir 5′-(L-Alaninyl)phosphate Disodium Salt (*N*-[({(1*S*,4*R*)-4-[2-Amino-6-(cyclopropylamino)-9*H*-purin-9-yl]cyclopent-2-en-1-yl}methoxy)phosphoryl]-D-alaninate Disodium Salt) (11). Abacavir 5′-[phenyl(methoxy-L-alaninyl)]phosphate (1:1 mixture of isomers of **3a** and **3b**, 500 mg, 0.95 mmol) was suspended in a solution of triethylamine (30 mL) and deionized water (30 mL) and stirred at room temperature for 18 h. The resulting solution was evaporated to dryness in vacuo, and the residue was dissolved in water (50 mL), extracted with CH_2Cl_2 (2 × 50 mL), and purified by anion exchange chromatography using a Sep-Pak Vac 35 cm^3 Accell Plus QMA cartridge (Waters Corp., P/N WAT054725) (HCO_3^- form) with an aqueous ammonium bicarbonate buffer (0−0.5 M linear gradient, 1 L). The appropriate fractions were combined and evaporated to dryness in vacuo. The residue was twice dissolved in deionized water and evaporated to dryness in vacuo to give **11** as the ammonium salt. This salt was dissolved in deionized water and passed through a Sep-Pak Vac 20 cm^3 Accell Plus CM cartridge (Waters Corp., P/N WAT054675) (Na^+ form) using deionized water. The appropriate fractions were combined and lyophilized to give 0.430 g (86% yield) of **11** disodium salt 2.5 hydrate as a white solid. ^1H NMR (D_2O): δ 7.71 (1H, s), 6.11 (1H, m), 5.80 (1H, m), 5.32 (1H, m), 3.66 (2H, m), 3.41 (1H, m), 2.99 (1H, m), 2.68 (2H, m), 1.49 (1H, m), 1.12 (3H, d), 0.74 (2H, m), 0.54 (2H, m). ^{31}P NMR (D_2O): δ 8.68. MS (ES$^-$) *m/z* 436 (M − H). Anal. ($C_{17}H_{22}N_7Na_2O_5P{\cdot}2.5H_2O$) C, H, N.

Antiviral Activity Assays. The activity of the test compounds against HIV-1- and HIV-2 induced cytopathicity in CEM cell cultures was measured at day 4 as previously described.[21] The antiviral activity of the test compounds was estimated by microscopical examination of virus-induced giant cell formation. HIV was added at approximately 100 $CCID_{50}$ to the cell cultures (1 $CCID_{50}$ = 50% cell culture infective dose). The anti-HSV-1 and anti-HSV-2 assays were based on inhibition of virus-induced cytopathicity in human embryonic lung (HEL) cell cultures. Confluent cell cultures in microtiter 96-well plates were inoculated with 100 $CCID_{50}$ of HSV-1 (KOS), HSV-2 (G), and HSV-1 TK^- (B2006 and VMW-1837) (1 $CCID_{50}$ being the virus dose to infect 50% of the cell cultures). After a 1 h virus adsorption period, residual virus was removed and the cell cultures were incubated in the presence of varying concentrations of the test compounds. Virus cytopathicity was recorded as soon as it reached completion in the control-virus-infected cell cultures.

The activity of the test compounds against HBV and the potential of these compounds to inhibit growth of human hepatoma cells were determined as previously described[26] using HepG2 2.2.15 cells, which constitutively produce HBV.

Radiolabeled Abacavir and Abacavir ProTide. [8-^{3}H]-Abacavir ([^{3}H]ABC) and [8-^{3}H]abacavir 5′-[phenyl(methoxy-L-alaninyl)]phosphate ([^{3}H]ABCpro) were prepared by Amersham Pharmacia Biotech (Buckinghamshire, England) via radiolabel exchange from tritiated water to ABC (**1**) and ABCpro (**3**), respectively. The radiolabel purity of these compounds was ≥95%. Their specific activities were approximately 7 and 9 Ci/mmol, respectively.

Metabolism of Abacavir and Its Aryloxymethoxyalaninylphosphoramidate Derivative in CEM Cells. The metabolism of [^{3}H]ABC and its radiolabeled ProTide aryloxymethylalaninyl abacavir monophosphate ([^{3}H]ABCpro) (**3**) in CEM cells was studied according to previously established procedures.[21] Briefly, human T4 lymphocyte CEM cells were seeded at $(2-4) \times 10^5$ cells/mL in RPMI-1640 culture medium supplemented with 10% fetal calf serum, 2 mM l-glutamine, and 0.075% $NaHCO_3$. Five milliliter cell suspensions in 25 cm^2 culture flasks were then incubated with 10 μM [^{3}H]ABC or 1.1 μM pro[^{3}H]ABC (5 μCi/5 mL). At 24 h, drug-treated cells were centrifuged, washed twice with cold RPMI-1640 medium, and precipitated with cold methanol/water (60:40). After centrifugation, the supernatants were subjected to HPLC analysis and separated on a Partisphere SAX column (Whatman, Clifton, NJ). A linear gradient of 0.005 M $(NH_4)H_2PO_4$ (pH 5.0) (buffer A) to 0.30 M $(NH_4)H_2PO_4$ (pH 5.0) (buffer B) was used. The retention times of the metabolites were as follows: ABC, carbovir (CBV), and pro-ABC, 2−3 min; ABCMP (abacavir 5′-monophosphate), 7−8 min; CBVMP (carbovir 5′-monophosphate) and alaninyl ABC-MP, 11−13 min; CBVDP (carbovir 5′-diphosphate), 19−21 min; CBVTP (carbovir 5′-triphosphate), 35−38 min.

Anabolism in HepG2 2.2.15 Cells. Anabolism studies with [^{3}H]ABC and [^{3}H]ABCpro in HepG2 2.2.15 cells were conducted generally as described by Paff et al.[25] In brief, HepG2 2.2.15 cells were seeded into T-75 flasks at 10^7 cells/flask, and after approximately 24 h of growth, the cultures were treated with [^{3}H]ABC or [^{3}H]ABCpro at the desired final concentration (0.01−100 μM, 2 mCi/μmol). At selected times following drug addition (2−48 h), cells were washed twice with ice-cold PBS (5 mL) and then extracted in situ by addition of ice-cold water (0.6 mL) followed by ice-cold acetonitrile (2.4 mL). Cell counts at collection were determined in representative flasks prior to extraction. The cell extracts were centrifuged, the supernatants were evaporated to dryness, and the dried extracts were reconstituted in 100 μL of HPLC-grade water. The reconstituted cell extracts were analyzed by strong anion-exchange HPLC according to the method described by Faletto et al.[14]

Pharmacokinetics and Oral Bioavailability in Cynomolgus Monkeys. An intravenous−oral crossover pharmacokinetic study was conducted with abacavir 5′-[phenyl(ethoxy-L-alaninyl)]phosphate succinate salt (**6** succinate) in cynomolgus monkeys. The in-life portion of this study was conducted at Covance Laboratories Inc. (Vienna, VA) under the oversight of its Institutional Animal Care and Use Committee. In brief, **6** succinate (2.3 mg/mL in 10% DMSO/90% saline) was administered intravenously and orally to three female cynomolgus monkeys (2.0−3.2 kg) at 11.5 mg/kg (5 mL/kg) with a 1-week washout period between doses (i.e., intravenous dose on day 1 and oral dose on day 8). The animals were fasted overnight prior to and until approximately 4 h after each dose; water was provided ad libitum. Serial blood samples were collected at selected times from predose to 24 h postdose and were centrifuged to obtain plasma within 30 min of collection. The resulting plasma samples were frozen at −20 °C and shipped on dry ice to GlaxoSmithKline for analysis.

Plasma samples (200 μL) were deproteinated by addition of approximately 4 volumes of acetonitrile (750 μL) followed by centrifugation (15800*g*, 10 min, 4 °C). The resulting supernatants were dried under nitrogen, and the dried extracts were reconstituted in 5% acetonitrile/0.1% acetic acid, pH 5.6 (200 μL). Plasma standards prepared by serial dilution of analyte stock solutions with normal monkey plasma were processed identically. The plasma extracts were analyzed by LC/MS/MS using a Phenomenex Luna 3-μm C18 column (100 mm × 2 mm). The samples were eluted by 5% acetonitrile/0.1% acetic acid, pH 5.6, for 3 min followed by a linear gradient to 95% acetonitrile over 5 min. Analytes were detected by positive ion MRM analysis, monitoring transitions *m/z* 542−191 for the ProTide (**6**), *m/z* 438−191 for alaninyl ABC-MP (**11**), *m/z* 367−191 for ABC-MP, *m/z* 287−191 for ABC (**1**), and *m/z* 248−152 for CBV (**2**). Analyte concentrations were determined by reference to a corresponding standard curve constructed by linear regression analysis of weighted ($1/x^2$) peak areas as measured by LC/MS analysis. Pharmacokinetic parameter values were determined by noncompartmental analysis of the plasma concentration−time data.

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Supporting Information Available: Analytical data for target compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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