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ARTICLE

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Reactive Aldehyde Metabolites from the Anti-HIV Drug Abacavir: Amino Acid Adducts as Possible Factors in Abacavir Toxicity

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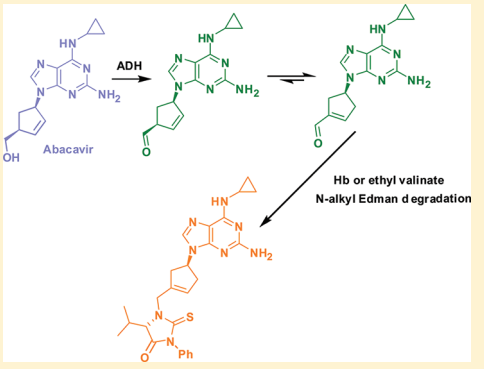
Supporting Information

ABSTRACT: Abacavir is a nucleoside reverse transcriptase inhibitor marketed since 1999 for the treatment of infection with the human immunodeficiency virus type 1 (HIV). Despite its clinical efficacy, abacavir administration has been associated with serious and sometimes fatal toxic events. Abacavir has been reported to undergo bioactivation *in vitro*, yielding reactive species that bind covalently to human serum albumin, but the haptenation mechanism and its significance to the toxic events induced by this anti-HIV drug have yet to be elucidated. Abacavir is extensively metabolized in the liver, resulting in inactive glucuronide and carboxylate metabolites. The metabolism of abacavir to the carboxylate involves a two-step oxidation via an unconjugated aldehyde, which under dehydrogenase activity isomerizes to a conjugated aldehyde. Concurrently with metabolic oxidation, the two putative aldehyde metabolites may be trapped by nucleophilic side groups in proteins yielding covalent adducts, which can be at the onset of the toxic events associated with abacavir. To gain insight into the role of aldehyde metabolites in abacavir-induced toxicity and with the ultimate goal of preparing reliable and fully characterized prospective biomarkers of exposure to the drug, we synthesized the two putative abacavir aldehyde metabolites and investigated their reaction with the α-amino group of valine. The resulting adducts were subsequently stabilized by reduction with sodium cyanoborohydride and derivatized with phenyl isothiocyanate, leading in both instances to the formation of the same phenylthiohydantoin, which was fully characterized by NMR and MS. These results suggest that the unconjugated aldehyde, initially formed *in vivo*, rapidly isomerizes to the thermodynamically more stable conjugated aldehyde, which is the electrophilic intermediate mainly involved in reaction with bionucleophiles. Moreover, we demonstrated that the reaction of the conjugated aldehyde with nitrogen bionucleophiles occurs exclusively via Schiff base formation, whereas soft sulfur nucleophiles react by Michael-type 1,4-addition to the α,β-unsaturated system. The synthetic phenylthiohydantoin adduct was subsequently used as standard for LC-ESI-MS monitoring of N-terminal valine adduct formation, upon modification of human hemoglobin *in vitro* with the conjugated abacavir aldehyde, followed by reduction and Edman degradation. The same postmodification strategy was applied to investigate the products formed by incubation of abacavir with rat liver cytosol, followed by trapping with ethyl valinate. In both instances, the major adduct detected corresponded to the synthetic phenylthiohydantoin standard. These results suggest that abacavir metabolism to the carboxylate(s) via aldehyde intermediate(s) could be a factor in the toxic events elicited by abacavir administration. Furthermore, the availability of a reliable and fully characterized synthetic standard of the abacavir adduct with the N-terminal valine of hemoglobin and its easy detection in the model hemoglobin modifications support the usefulness of this adduct as a prospective biomarker of abacavir toxicity in humans.



INTRODUCTION

Abacavir ({(1*S*,4*R*)-4-[2-amino-6-(cyclopropylamino)-9*H*-purin-9-yl]cyclopent-2-en-1-yl}methanol, **1**, Scheme 1) is a nucleoside reverse transcriptase inhibitor marketed since 1999 for the treatment of infection with the human immunodeficiency virus type 1 (HIV).[1] This NRTI is commercially available as abacavir sulfate (ZIAGEN) or as part of a three-drug regimen (Trizivir) and is used by both adults and children.[2]

Abacavir-based regimens have a significant role in HIV-treatment guidelines due to the antiretroviral efficacy of the drug and its availability in one-pill fixed-dose combinations.[3] Despite its clinical efficacy and although individual vulnerability to adverse effects differs among patients, abacavir administration is associated with toxic events. Potentially life-threatening abacavir-induced hypersensitivity reactions (HSRs) have been described to occur usually within the first six weeks of treatment, albeit with an incidence of less than 5%, and lead to discontinuation of the

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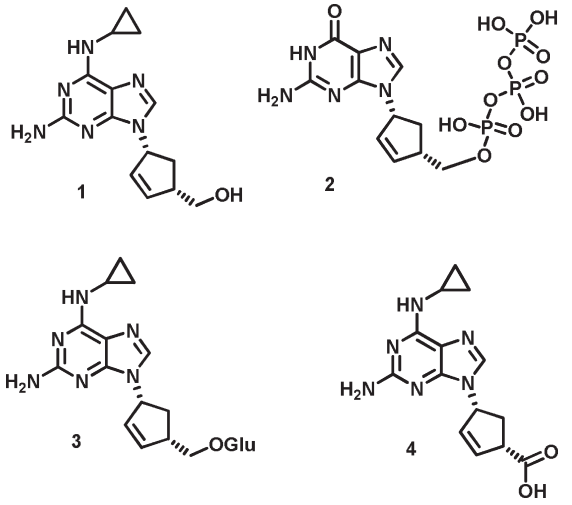
Scheme 1. Structures of Abacavir (1), Its Active Metabolite Carbovir Triphosphate (2), and the Two Inactive Metabolites, the Glucuronide (3) and the Carboxylate, Drawn As the Undissociated Acid (4)[a]



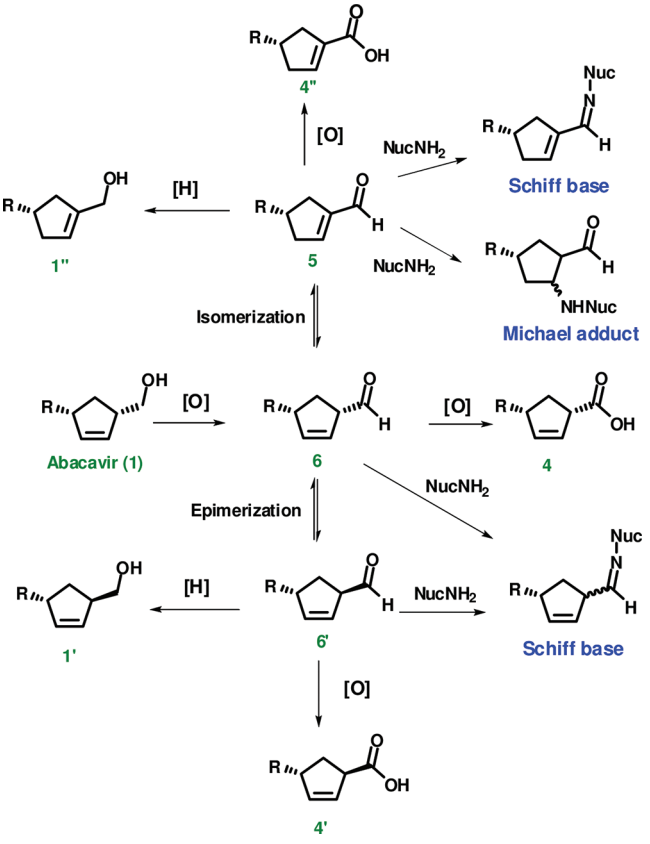
[a] Glu represents the glucuronic acid-derived substituent. Metabolites **3** and **4** are often designated in the literature as abacavir 5′-glucuronide and abacavir 5′-carboxylate (ionized form), respectively. These designations assume a numbering system for the cyclopentene ring analogous to that of ribose. In the present article, we have adopted IUPAC nomenclature, whereupon the cyclopentene carbon attached to the purine is numbered 4, rather than 1. To prevent ambiguity, the numerical label is omitted from the common designations of abacavir metabolites throughout the text.

drug in susceptible patients. These HSRs are characterized by a variety of symptoms, of which fever and skin rash are the most common.[4,5] Although an association between long-term abacavir exposure and increased cardiovascular risk is still controversial,[6,7] current guidelines recommend caution in administering abacavir to patients who are at high risk for cardiovascular disease.[3]

The recognition of an association between susceptibility to abacavir-induced HSRs and the presence of the human leukocyte allele (HLA)-B*5701, especially among Caucasians, has made possible a prospective test to detect allele-positive patients, who should not receive abacavir.[8] Despite the utility of this test for identifying individuals at greatest risk, it does not predict which patients will definitely experience HSRs.[9] Therefore, the development of reliable biomarkers of abacavir toxicity is essential, and comprehensive knowledge of the molecular mechanisms underlying the toxic events is of unquestionable significance for accurate risk/benefit estimations.

Metabolic activation to reactive metabolites plays an important role in drug-induced toxicity.[10] Abacavir is a prodrug that is converted to the active metabolite carbovir triphosphate (**2**, Scheme 1) via stepwise intracellular anabolism.[11] Concurrent with this activation process, abacavir is extensively metabolized in the liver via two pathways, mediated by uridine diphosphate glucuronyltransferase and alcohol dehydrogenase (ADH), which yield a glucuronide (**3**) and a carboxylate (**4**), respectively, as the major metabolites (Scheme 1). These metabolites are excreted primarily in the urine, where in combination they account for 66% of the dose; an additional 15% of the dose is converted into a number of minor metabolites,[12] but structural considerations

Scheme 2. Putative Metabolic Transformations from Abacavir, Involving Oxidation, Isomerization, and Epimerization Reactions[a]



[a] Protein-adduct formation can occur through the aldehyde intermediates, **5**, **6**, and **6′**; $NucNH_2$ represents a nucleophilic protein amino residue, leading to Schiff base formation or Michael addition by reaction with the aldehydes [adapted from Walsh et al.[13]]. Sulfur nucleophiles will react with aldehyde **5** through Michael addition. **R** represents the 6-cyclopropylaminopurine moiety.

suggest that none of those identified to date are potentially reactive intermediates. However, Walsh et al.[13] demonstrated that abacavir metabolism to the carboxylate **4** involves a two step oxidation process, via an aldehyde intermediate, which was shown to be mediated *in vitro* by human ADH. Although attempts to identify the aldehyde *in vivo* failed, in part due to difficulties in the preparation of a synthetic standard, these authors obtained indirect LC-MS evidence for its formation by incubating [^{14}C]abacavir with different human ADH isoforms, followed by derivatization with methoxylamine. Furthermore, the detection of the carboxylate in these incubations showed that aldehyde dehydrogenase (ALDH) involvement is not required for abacavir metabolism. Moreover, the formation of isomers of both the acid metabolite and the parent drug through ADH metabolism led Walsh et al.[13] to propose a metabolic pathway (Scheme 2) involving double bond migration and epimerization processes upon the formation of two putative aldehyde intermediates: an α,β-unsaturated derivative (**5**, conjugated aldehyde) and a β,γ-unsaturated species (**6**, unconjugated aldehyde). These authors also noted that ADH-mediated abacavir metabolism could result in covalent binding to human serum albumin. Nonetheless, the haptenation mechanism and its

significance to the toxic reactions induced by abacavir have yet to be elucidated.

As immune-mediated responses, drug-induced HSRs depend on the ability of drugs, or their metabolites, to bind covalently to proteins yielding drug−protein adducts that (unlike their parent drugs or metabolites) can interact directly with immune receptors, generating antigens.[14] Aldehydes are common haptens frequently implicated in allergenic responses.[15] Given that ADH is present in epithelial tissues, including the skin,[16] the involvement of aldehyde metabolites in abacavir-induced skin rash appears entirely plausible. Moreover, although the molecular mechanisms of drug-induced cardiotoxicity are much less understood than those of HSR,[17] several aldehydes have been associated with the onset of cardiovascular pathologies. For instance, acetaldehyde, the primary metabolic product of ethanol, is thought to be at the origin of alcoholic cardiomyopathy,[18] and genetic polymorphisms in ADH and ALDH appear to determine the susceptibility to alcoholism and alcohol-induced diseases.[19,20] Likewise, direct exposure to the highly reactive aldehyde acrolein, an endogenous product of lipid peroxidation and ubiquitous environmental pollutant, has been shown to cause myocardial dysfunction in mice, possibly due to site-specific protein modification.[21]

Aldehydes are short-lived species *in vivo*, a characteristic that makes them extremely difficult to detect; consequently, the establishment of direct correlations between aldehyde levels and the induction of specific pathologies is not straightforward. However, like other reactive electrophiles, aldehydes bind covalently to biomacromolecules, and the resulting adducts can be quantified in fluids and tissues. Typically, stable protein adducts are the most convenient biomarkers of exposure,[22] and the abundant blood protein, hemoglobin (Hb), is often used as an easily accessible model for biomonitoring reactive metabolites and elucidating the haptenation mechanisms of toxicants.[23−25]

With the goal of preparing reliable and fully characterized potential biomarkers of abacavir toxicity, and ultimately gaining insight into the molecular mechanisms of abacavir-induced pathologies, we synthesized the two putative aldehyde metabolites from abacavir and investigated their reactivity with the α-amino group of valine. One synthetic adduct was obtained and subsequently used as the standard to monitor adduct formation with Hb *in vitro*. The availability of this prospective biomarker should help clarify the significance of abacavir metabolism to aldehyde(s) and subsequent haptenation in the toxic events associated with the drug.

MATERIALS AND METHODS

Chemicals. Human Hb and all other commercially available reagents and enzymes were acquired from Sigma-Aldrich Química, S.A. (Madrid, Spain), unless specified otherwise, and used as received. L-Amino acids were used in all instances. Whenever necessary, solvents were purified by standard methods.[26]

Instrumentation. *HPLC.* Analytical and semipreparative HPLC was conducted on an Ultimate 3000 Dionex system, consisting of an LPG-3400A quaternary gradient pump and a diode array spectrophotometric detector (Dionex Co., Sunnyvale, CA), and equipped with a Rheodyne model 8125 injector (Rheodyne, Rohnert Park, CA). HPLC analyses were performed with a Luna C18 (2) column (250 mm × 4.6 mm; 5 μm; Phenomenex, Torrance, CA), at a flow rate of 1 mL/min. Semipreparative HPLC separations were conducted with a Luna C18 (2) column (250 mm × 10 mm; 5 μm; Phenomenex) at a flow rate of 3 mL/min. The UV absorbance was monitored at 254 nm.

Liquid Chromatography−Electrospray Ionization−Tandem Mass Spectrometry (LC-ESI-MS/MS). LC-ESI-MS/MS analyses were performed with a ProStar 410 autosampler, two 210-LC chromatography pumps, a ProStar 335 diode array detector, and a 500-MS ion trap mass spectrometer, with an ESI ion source (Varian, Inc., Palo Alto, CA). Data acquisition and processing were performed using Varian MS Control 6.9 software. The samples were injected onto the column via a Rheodyne injector with a 20 μL loop. Separations were conducted at 30 °C, using a Luna C18 (2) column (150 mm × 2 mm, 3 μm; Phenomenex, Torrance, CA). The mobile phase was delivered at a flow rate of 200 μL/min, using a 5-min isocratic elution with 5% acetonitrile in 0.1% aqueous formic acid, followed by a 30-min linear gradient from 5 to 70% acetonitrile, a 2-min linear gradient to 100% acetonitrile, and an 8-min isocratic elution with acetonitrile. For the analysis of samples subjected to N-alkyl Edman degradation, the elution was conducted with a 15-min linear gradient from 0 to 100% acetonitrile in 0.1% formic acid, followed by a 2-min isocratic elution with acetonitrile, and an 8-min linear gradient back to 100% of 0.1% formic acid. The mass spectrometer was operated in the positive ESI mode; the optimized operating parameters were ion spray voltage, +5.2 kV; capillary voltage, 80 V; and RF loading, 70%. Nitrogen was used as the nebulizing and drying gas, at pressures of 50 and 30 psi, respectively; the drying gas temperature was 350 °C. MS/MS spectra were obtained with an isolation window of 2 Da, excitation energy values between 0.9 and 1.7 V, and an excitation time of 10 ms.

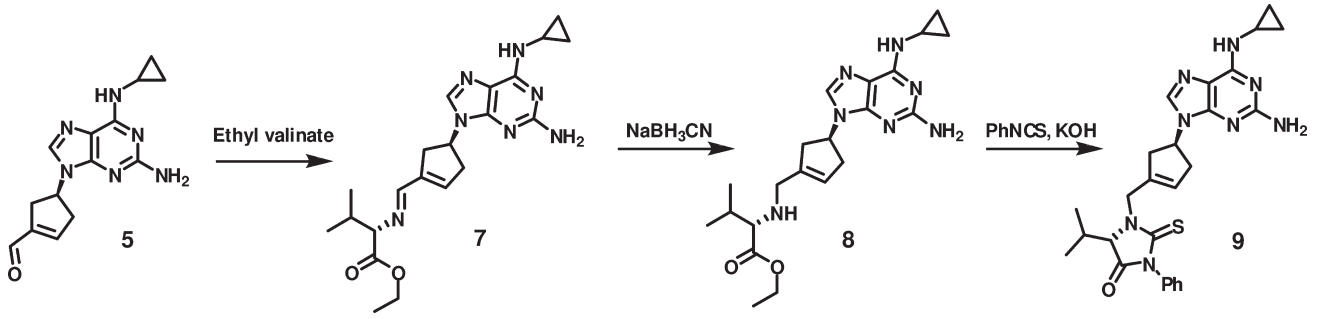
NMR. ^{1}H NMR spectra were recorded on Bruker Avance III 400 or 500 spectrometers, operating at 400 and 500 MHz, respectively. ^{13}C NMR spectra were recorded on the same instruments, operating at 100.62 and 125.77 MHz, respectively. Chemical shifts are reported in ppm downfield from tetramethylsilane, and coupling constants (J) are reported in Hz. Geminal protons are denoted with "a" and "b" subscripts. The presence of labile protons was confirmed by chemical exchange with D_2O. Resonance and structural assignments were based on the analysis of coupling patterns, including the ^{13}C−^{1}H coupling profiles obtained in bidimensional heteronuclear multiple bond correlation (HMBC) and heteronuclear multiple quantum coherence (HMQC) or heteronuclear single quantum coherence (HSQC) experiments, performed with Bruker standard pulse programs. Whenever necessary, distortionless enhancement by polarization transfer (DEPT) and ^{1}H−^{1}H bidimensional correlation spectroscopy (COSY) experiments were also perfomed to assist the structural assignments. ^{13}C resonances were not discriminated whenever small sample quantities precluded the recording of one-dimensional ^{13}C NMR spectra with good signal/noise ratios, despite having been detected in the inverse heteronuclear bidimensional experiments.

General Procedures. *Isolation of Rat Liver Cytosol.* Wistar adult rats were obtained from the *vivarium* of the Faculty of Medical Sciences, New University of Lisbon. The animal handling protocol was approved by the Institutional Animal Care and Use Committee.

Rat liver cytosol was isolated by differential centrifugation.[27] Briefly, the rats (ca. 200 g bw) were anesthetized with an ip injection of 60 mg/kg pentobarbital. The livers were excised immediately, perfused with ice-cold 0.15 M KCl, weighed, minced, and homogenized with cold 250 mM sucrose in 10 mM Tris-HCl, pH 7.4, at approximately 3 g of liver tissue per mL of sucrose buffer. The homogenate was centrifuged at 10,000g for 20 min at 4 °C. The supernatant was then centrifuged at 100,000g for 60 min at 4 °C. The final 100,000g supernatant (cytosol) and pellet (microsomes) were collected and stored separated at −80 °C.

N-Alkyl Edman Degradation.[28] Dried samples were dissolved in DMF (250 μL), followed by the addition of 1 M NaOH (10 μL) and phenyl isothiocyanate (1.5 μL, 58.6 μmol). The solution was subsequently stirred for 2 h at 37 °C and then for 1.5 h at 45 °C. Upon cooling to room temperature, water (2.5 mL) was added, and the adducts were

Scheme 3. Synthesis of Hydantoin 9 from Conjugated Abacavir Aldehyde 5[a]



[a] The synthetic sequence involves the initial formation of the Schiff base **7** by reaction of **5** with ethyl valinate, followed by stabilization of **7** as the reduced amine **8**, and final derivatization of **8** with phenyl isothiocyanate in basic medium.

extracted with ethyl acetate (2 × 2.5 mL). The organic phase was dried under reduced pressure, and the contents were purified by semipreparative HPLC and/or analyzed by LC-ESI-MS/MS.

Syntheses. *Preparation of Abacavir Aldehydes. (4S)-4-[2-Amino-6-(cyclopropylamino)-9H-purin-9-yl]cyclopent-1-ene-1-carbaldehyde (**5**).* Dry DMSO (2.6 equiv, 130 μL, 1.83 mmol) was added to a solution of oxalyl chloride (1.3 equiv, 78 μL, 0.91 mmol) in dry THF (3 mL), under nitrogen at −78 °C. The resulting mixture was kept under stirring for 5 min, a suspension of abacavir (**1**, 1.0 equiv, 200 mg, 0.70 mmol) in dry THF (9 mL) was then added and stirring was continued at −78 °C for 1 h. At this stage, anhydrous triethylamine (5.2 equiv, 507 μL, 3.64 mmol) was added to the mixture, the temperature was allowed to rise to 0 °C, and stirring was maintained for 1 h. Following the addition of water (9 mL), the mixture was extracted with diethyl ether (3 × 100 mL), and the combined organic phases were dried over anhydrous magnesium sulfate. The resulting mixture was purified by preparative TLC on silica [CH_2Cl_2/MeOH (9:1)] affording a yellow oil (36 mg, 18%). ^{1}H NMR ($CDCl_3$): δ (ppm) 9.77 (1H, s, aldehyde HCO), 7.38 (1H, s, purine H8), 6.89−6.87 (1H, m, cyclopentene H2), 5.95 (1H, m, purine N^6H), 5.16−5.14 (1H, m, cyclopentene H4), 4.90 (2H, s, purine N^2H_2), 3.23−3.02 (2H, m, cyclopentene $H3_a$ + cyclopentene $H5_a$), 2.97−2.88 (3H, m, cyclopentene $H3_b$ + cyclopentene $H5_b$ + cyclopropyl H10), 0.77−0.75 (2H, m, cyclopropyl $H11_a$ + cyclopropyl $H12_a$), 0.52−0.51 (2H, m, cyclopropyl $H11_b$ + cyclopropyl $H12_b$). ^{13}C NMR ($CDCl_3$): δ (ppm) 189.0 (C═O), 160.2 (purine C2/C6), 156.6 (purine C2/C6), 151.2 (purine C4), 148.8 (cyclopentene C2), 145.6 (cyclopentene C1), 135.1 (purine C8), 115.0 (purine C5), 52.5 (cyclopentene C4), 42.3 (cyclopentene C3), 36.1 (cyclopentene C5), 23.9 (cyclopropyl C10), 7.6 (cyclopropyl C11 + C12). MS (ESI): *m*/*z* 285 $[MH]^+$, 191 $[purineH]^+$.

*(4R)-4-[2-Amino-6-(cyclopropylamino)-9H-purin-9-yl]cyclopent-2-ene-1-carbaldehyde (**6**/**6′**).* Dry DMSO (4.0 equiv, 100 μL, 1.41 mmol) was added to a solution of oxalyl chloride (2.0 equiv, 60 μL, 0.70 mmol) in dry THF (3 mL), under nitrogen at −78 °C. The resulting mixture was kept under stirring for 5 min, a suspension of abacavir (**1**, 1.0 equiv, 100 mg, 0.35 mmol) in dry THF (6 mL) was then added, and stirring was continued at −78 °C for 1 h. At this stage, *N*,*N*-diisopropylethylamine (5.0 equiv, 304 μL, 1.75 mmol) was added to the mixture, the temperature was allowed to rise to 0 °C, and stirring was maintained for 1 h. Following the addition of water (9 mL), the mixture was extracted with diethyl ether (3 × 100 mL), and the combined organic phases were dried over anhydrous magnesium sulfate. The resulting mixture was purified by preparative TLC on silica [CH_2Cl_2/MeOH (10:1)] affording a yellow oil (14 mg, 14%). ^{1}H NMR ($CDCl_3$): δ (ppm) 9.83 (1H, s, aldehyde HCO), 7.48 (1H, s, purine H8), 6.11−6.08 (1H, m, cyclopentene H3), 5.95 (1H, m, purine N^6H), 5.79−5.77 (1H, m, cyclopentene H2), 5.47−5.42 (1H, m, cyclopentene H4), 4.79 (2H, s, purine N^2H_2), 3.11−3.07 (1H, m, cyclopentene H1), 2.98−2.94 (1H, m, cyclopropyl H10), 2.81−2.70 (1H, m, cyclopentene $H5_a$), 2.09−2.00 (1H, m, cyclopentene $H5_b$), 0.85−0.80 (2H, m, cyclopropyl $CH11_a$ + cyclopropyl $CH12_a$), 0.62−0.58 (2H, m, cyclopropyl $CH11_b$ + cyclopropyl $CH12_b$). MS (ESI): *m*/*z* 285 $[MH]^+$, 191 $[purineH]^+$.

*Reaction of Ethyl Valinate with Abacavir Aldehydes. Reaction with Aldehyde **5**.* A solution of ethyl valinate hydrochloride (3.9 equiv, 20.0 mg, 110 μmol), was prepared in 50 mM phosphate, pH 7.4 (400 μL), and subsequently treated with sodium hydrogen carbonate (3.9 equiv, 9.0 mg, 110.1 μmol) for 30 min. A solution of **5** (1.0 equiv, 8.0 mg, 28.2 μmol) in THF (1.0 mL) was then added. The resulting mixture was incubated at 37 °C for 30 min, whereupon it was treated with sodium cyanoborohydride (21.0 mg, 334.2 μmol) and then incubated overnight at 37 °C. After solvent removal under reduced pressure, the mixture was subjected to an N-alkyl Edman procedure (*vide supra*). Following the concentration of the organic phase under reduced pressure, the mixture was purified by semipreparative HPLC using a 15-min linear gradient from 0 to 100% acetonitrile in 0.1% formic acid, followed by a 2-min isocratic elution with acetonitrile and an 8-min linear gradient back to 100% of 0.1% formic acid.

*(5S)-1-{[(4S)-4-(2-Amino-6-(cyclopropylamino)-9H-purin-9-yl)-cyclopent-1-en-1-yl]methyl}-3-phenyl-5-(propan-2-yl)-2-thioxoimidazolidin-4-one (**9**, Scheme 3).* Compound **9** was isolated in 15% yield (2.1 mg). Retention time: 11 min. UV: λ_{max} 236, 264, 296 nm. ^{1}H NMR (methanol-d_4): δ (ppm) 7.79 (1H, s, purine H8), 7.49−7.41 (3H, m, phenyl H3,5 + phenyl H4), 7.23 (2H, d, *J* = 7.5, phenyl H2,6), 5.87 (1H, s, cyclopentene H2), 5.17−5.14 (2H, m, cyclopentene H4 + hydantoin $H9_a$), 4.27−4.26 (2H, m, hydantoin H5 + hydantoin $H9_b$), 3.05−2.96 (2H, m, cyclopentene $H3_a$ + cyclopentene $H5_a$), 2.90 (1H, bs, cyclopropyl H10), 2.78−2.75 (1H, m, cyclopentene $H5_b$), 2.68−2.64 (1H, m, cyclopentene $H3_b$), 2.55−2.47 (1H, m, isopropyl CH), 1.24 (3H, d, *J* = 7.0, isopropyl CH_3), 0.94 (3H, d, *J* = 6.8, isopropyl CH_3), 0.88−0.84 (2H, m, cyclopropyl $H11_a$ + cyclopropyl $H12_a$), 0.64−0.61 (2H, m, cyclopropyl $H11_b$ + cyclopropyl $CH11_b$). ^{13}C NMR (methanol-d_4): δ (ppm): 183.0 (hydantoin C═S), 172.5 (hydantoin C═O), 159.6 (purine C2/C6), 155.6 (purine C2/C6), 150.4 (purine C4), 137.0 (cyclopentene C1), 136.1 (purine C8), 133.9 (phenyl Cipso), 128.6 (phenyl C), 128.5 (phenyl C), 126.8 (cyclopentene C2), 113.1 (purine C5), 66.2 (hydantoin C5), 53.3 (cyclopentene C4), 44.5 (hydantoin C9), 40.2 (cyclopentene C3), 38.7 (cyclopentene C5), 28.9 (isopropyl CH), 22.9 (cyclopropyl C10), 16.4 (isopropyl CH_3), 14.6 (isopropyl CH_3), 6.2 (cyclopropyl C11 + cyclopropyl C12). MS (ESI): *m*/*z* 503 $[MH]^+$, 313 [MH − purine moiety]$^+$, 235 [hydantoin H]$^+$, 191 [purine H] $^+$.

*Reaction with Aldehyde **6**/**6′**.* The reaction with aldehyde **6**/**6′** (10.0 mg, 35.2 μmol) was conducted as described for aldehyde **5**. The N-alkyl

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Edman adduct **9** was obtained in 3% yield (0.5 mg). For spectroscopic data, *vide supra*.

*Reaction of Lysine with the Conjugated Aldehyde **5***. A solution of **5** (1.0 equiv, 8.0 mg, 28.2 μmol), in THF (400 μL), was added to a solution of lysine (8.0 equiv, 34.0 mg, 230 μmol) in 50 mM phosphate at pH 7.4 (1 mL). Following incubation at 37 °C for 30 min, sodium cyanoborohydride (3.0 mg, 47.7 μmol) was added, and the incubation was continued overnight at 37 °C. The mixture was purified by semipreparative HPLC, using a 10-min linear gradient from 0 to 5% acetonitrile in 0.1% formic acid, followed by a 10-min linear gradient from 5 to 10% acetonitrile in 0.1% formic acid. A small amount of a product with retention time 10.6 min and consistent with a lysine adduct was isolated (≤0.1 mg). MS (ESI): m/z 415 $[MH]^+$, 286 [MH − aminohexanoic acid]$^+$, 208 $[MH_2]^{2+}$.

*Reaction of Cysteine with Conjugated Aldehyde **5***. A solution of **5** (1.0 equiv, 4.5 mg, 15.8 μmol) in THF (500 μL) was reacted with a solution of cysteine (4.0 equiv, 7.7 mg, 63.6 μmol,) in 50 mM phosphate, pH 7.4 (300 μL). The resulting mixture was incubated overnight at 37 °C and then analyzed by LC-ESI-MS/MS.

*Modification of Human Hemoglobin with Conjugated Aldehyde **5***. The typical modification conditions were as follows: to a solution of human Hb (7.5 mg) in 50 mM phosphate, pH 7.4 (7.5 mL), was added a solution of **5** (7.5 mg, 26.4 μmol) in THF (250 μL). Following incubation at 37 °C for 30 or 60 min, sodium cyanoborohydride (17 mg, 270.5 μmol) was added, and the incubation was continued overnight at 37 °C. The solvent was then removed under vacuum, and the mixture was subjected to N-alkyl Edman degradation (*vide supra*). The resulting residue was analyzed by LC-ESI-MS/MS.

In a parallel experiment, two 1.5 mL aliquots were removed after incubation at 37 °C for 30 min. The reduction step with sodium cyanoborohydride was omitted for one of the aliquots but performed in the other. Both aliquots were subsequently hydrolyzed to amino acids with Pronase E and leucine aminopeptidase M, as described in ref 29. The enzymatic hydrolysates were then analyzed directly by LC-ESI-MS/MS.

Incubation of Abacavir with Rat Liver Cytosol. Rat liver cytosol (1 mg/mL; 900 μL) in 50 mM sodium pyrophosphate, pH 8.8, was added to an aqueous solution of abacavir (33 μg/mL; 100 μL). Following the addition of 75 mM NAD^+ in 50 mM sodium pyrophosphate, pH 8.8 (100 μL), the mixture was incubated at 37 °C under gentle stirring. Negative control incubations were conducted in the same manner using cytosol samples pretreated at 60 °C for 1 h, to ensure protein denaturation. The incubations were run in triplicate. In each instance, two 500 μL aliquots were removed, one after 2 h and the other after 20 h. Upon cooling, each aliquot was treated with a solution of ethyl valinate hydrochloride (460 μM, 100 μL) in sodium pyrophosphate, pH 8.8. After incubation at 37 °C for 30 min, sodium cyanoborohydride (100 μg) was added to the mixture, and the incubation was continued overnight. Following filtration (Amicon 5K, 0.5 mL) under centrifugation (15 min, at 14,000*g* and 4 °C), the samples were dried under vacuum and subjected to an N-alkyl Edman procedure (*vide supra*). The organic phase was dried under vacuum, and the samples were redissolved in methanol (200 μL) and analyzed by LC-ESI-MS/MS.

RESULTS AND DISCUSSION

Aldehyde Synthesis and Characterization. *Synthesis of Aldehydes **5** and **6***. The synthesis of an aldehyde from a primary alcohol is usually a standard procedure, and multiple oxidation methodologies are available for this functional group interconversion.[30] However, the oxidation of abacavir to its aldehyde derivative(s) proved to be considerably more demanding than initially anticipated, which may explain why previous attempts[13] have failed. Although we tested several oxidation strategies,[30−34] the conjugated aldehyde **5** (Scheme 2) was only conveniently prepared upon abacavir oxidation under Swern conditions in THF, using triethylamine as the base.[31] We found the reaction efficiency to be extremely affected by minor experimental variations, including the choice of solvent. Of note, although dichloromethane is the typical solvent for Swern oxidations,[30] we only isolated trace amounts of aldehyde when using this solvent. Under optimized conditions (cf. Materials and Methods), we still obtained a complex mixture, and the yield of aldehyde **5** did not exceed 18%.

The formation of aldehyde **5** from abacavir stems from the occurrence of double bond isomerization concomitantly with the oxidation of the primary alcohol. This type of isomerization has been reported for other substrates under Swern oxidation conditions, when triethylamine is used as the base,[35] and can be explained by the formation of a resonance-stabilized allylic enolate, which reprotonates to the thermodinamically more stable α,β-unsaturated aldehyde (**5**) rather than its β,γ-unsaturated isomer (**6**/**6**′, henceforth designated as **6**; Scheme 2). Nonetheless, the use of a more sterically hindered base, *N*,*N*-diisopropylethylamine, allowed us to prevent enolate formation, at least in part, to obtain the β,γ-unsaturated aldehyde (**6**) in 14% yield. However, it should be pointed out that even using optimized conditions for the formation of **6** (cf. Materials and Methods) a nonquantified amount of **5** was always detected during the preparative TLC separation. Isomerization to the α,β-unsaturated aldehyde may have occurred either with base-catalysis, prior to workup, or acid-catalysis, during the chromatography step on silica.

*Structural Characterization of Aldehydes **5** and **6***. Structural evidence for the formation of each of the two isomeric aldehydes was initially obtained by mass spectrometry. Both aldehydes had ESI mass spectra showing the protonated molecule at m/z 285 and a fragment ion at m/z 191, stemming from the loss of the cyclopentenylcarbaldehyde moieties. Moreover, the ^{1}H NMR spectra of the conjugated (**5**) and unconjugated (**6**) aldehyde clearly showed singlets at 9.77 and 9.83 ppm, respectively. These signals had ^{1}H−^{13}C HSQC correlations with ^{13}C NMR carbonyl resonances (both at 189.0 ppm), which clearly indicate the presence of an aldehyde group in each of the molecules. Likewise, the ^{1}H NMR and ^{13}C NMR spectra clearly showed the resonances expected from the cyclopropyl and purine moieties. The major differences in the NMR resonances of the two isomeric aldehydes were observed for the cyclopentene ring due to the distinct locations of the double bond. Possibly due to isomerization in solution (*vide infra*), it was not possible to obtain ^{13}C NMR spectra with good signal/noise ratio for aldehyde **6**. Nonetheless, carbon resonance assignments could be performed by analysis of the correlations observed in the inverse bidimensional HSQC and HMBC experiments. Thus, the structures of the two aldehydes were discriminated on the basis of the results obtained with HSQC, HMBC, COSY, and DEPT NMR experiments.

The resonances of the cyclopentene moiety of **6** (cf. Materials and Methods) and abacavir (not shown) displayed very similar ^{1}H NMR and ^{13}C NMR spectral profiles, suggesting an identical location for the double bond (i.e., clear evidence for two tertiary olefinic carbons and a single secondary carbon). The ^{13}C NMR spectrum of the conjugated aldehyde **5** showed a very different pattern, exhibiting signals from two secondary carbons at 36.1 (C5) and 42.3 (C3) ppm and two olefinic carbons, of which one was tertiary (C2, at 148.8 ppm) and the other was quaternary

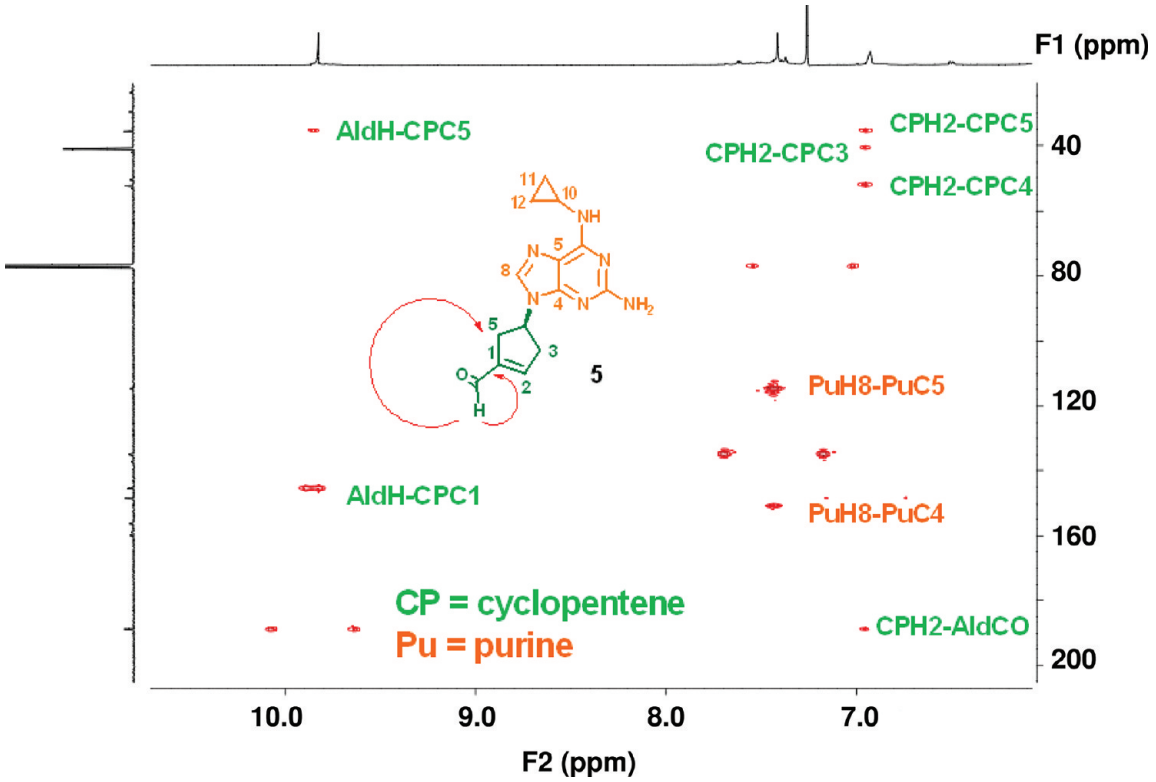


Figure 1. Expanded region of the $^{1}H-^{13}C$ HMBC spectrum of aldehyde **5**, displaying the connectivities between the aldehyde (H−C═O) proton and carbons C1 and C5 of the cyclopentene moiety.

(C1, at 145.6 ppm); this pattern per se allowed the unequivocal assignment of the double bond location between C1 and C2 of the cyclopentene moiety. Conclusive evidence for the structural assignment of conjugated aldehyde **5** stemmed from the observation of the two- and three-bond HMBC correlations of the aldehyde proton (at 9.77 ppm) with the quaternary olefinic carbon at 145.6 ppm (C1) and the secondary carbon at 36.1 ppm (C5), respectively (Figure 1).

It should be noted that whereas the NMR spectra of the isolated aldehydes **5** and **6**, recorded in deuterochloroform, did not provide evidence for the presence of any identifiable contaminants, LC-ESI-MS analysis of methanol solutions of the same compounds revealed the existence of hydrophobic impurities with high molecular mass. Although we have not succeeded in their characterization, it is plausible that these species arose from polymerization reactions of the aldehydes. Moreover, during the LC-ESI-MS analysis of aldehyde **6**, we also confirmed that this compound isomerizes to **5** in methanol solution. These observations indicate that both aldehydes are short-lived species in solution. In spite of this, neither the structural characterizations discussed above nor any subsequent synthetic procedures using the aldehydes as starting materials (*vide infra*) were compromised. At most, the presence of small amounts of unquantified aldehyde-derived impurities in solution caused slight underestimations of the synthetic adduct yields reported below.

Reactions of the Abacavir Aldehydes with Ethyl Valinate. N-Terminal valine residues in Hb are primary sites of reaction with several classes of electrophiles, including aldehydes.[36] Moreover, the possibility of selectively detaching adducts to the N-terminal residues using the N-alkyl Edman method, a mild, simple, wide-ranging, and sensitive postmodification procedure, explains the extensive use of Hb valine adducts as biomarkers of toxicity.[37]

We started by performing incubations of the synthetic aldehydes **5** and **6** with ethyl valinate, with the purpose of obtaining fully characterized adduct standards that could subsequently be used to monitor aldehyde-promoted Hb modifications.

Furthermore, the structural characterization of these standards was expected to help clarify the chemical mechanisms and regioselectivities potentially involved in the haptenation of abacavir aldehyde(s) to cellular proteins. In fact, as pointed out by Walsh et al.,[13] both aldehydes could conceivably react with nitrogen nucleophiles (e.g., ethyl valinate) via Schiff base formation, but Michael-type 1,4-addition to the α,β-unsaturated system of aldehyde **5** (Scheme 2) is also plausible.

The incubations were conducted at 37 °C, following the addition of a THF solution of the aldehyde to a solution of ethyl valinate in 50 mM phosphate buffer (pH 7.4), using a 4-fold molar excess of the nucleophile. Because of the expected formation of a Schiff base, which should be prone to hydrolysis, the mixtures were subsequently treated with sodium cyanoborohydride to stabilize the imino groups by selective reduction.

*Reaction of Aldehyde **5** with Ethyl Valinate.* LC-ESI/MS analysis (Figure 2) of the reaction mixture obtained in incubations with the α,β-unsaturated aldehyde **5** indicated the presence of an adduct consistent with initial formation of the Schiff base **7**, which upon reduction yielded the detected adduct **8** (Scheme 3). The extracted ion chromatogram (m/z 414) clearly showed a signal at 24.6 min under the chromatographic conditions used (Figure 2). The m/z value for the corresponding protonated molecule provided unequivocal evidence for 1,2-addition to the

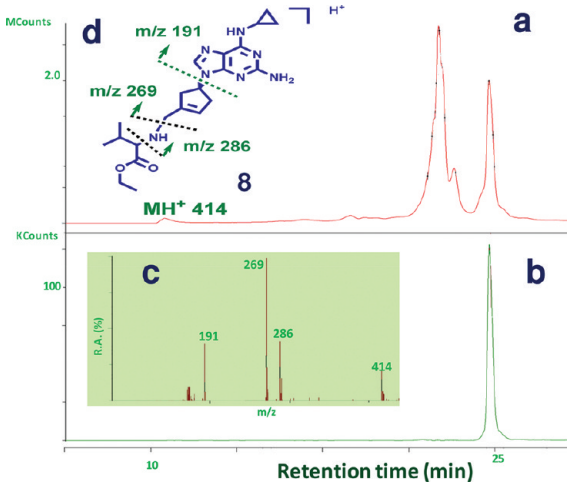


Figure 2. LC-ESI-MS analysis of adduct **8**, obtained from incubation of the α,β-unsaturated aldehyde **5** with ethyl valinate, followed by reduction with sodium cyanoborohydride: (a) total ion chromatogram; (b) extracted ion chromatogram (m/z 414); (c) mass spectrum of the protonated molecule; (d) proposed fragmentation pattern. The elution conditions are outlined in Materials and Methods.

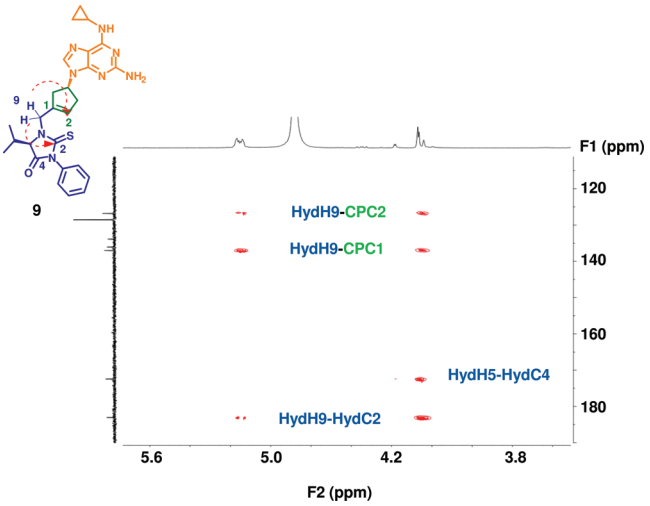


Figure 3. Expanded region of the $^{1}H-^{13}C$ HMBC spectrum of adduct **9**, highlighting the 3-bond correlations between the geminal methylene protons (labeled HydH9) and both the thiocarbonyl carbon of the thiohydantoin moiety (labeled HydC2) and the tertiary olefinic carbon of the cyclopentene ring (labeled CPC2).

carbonyl group of **5**, rather than Michael-type 1,4-addition. As shown in Figure 2, the fragment ions at m/z 286 (loss of ethyl 3-methylbutanoate from MH^+), m/z 269 (base peak; loss of ethyl valinate from MH^+), and m/z 191 (protonated purine moiety) fully support the structural assignment.

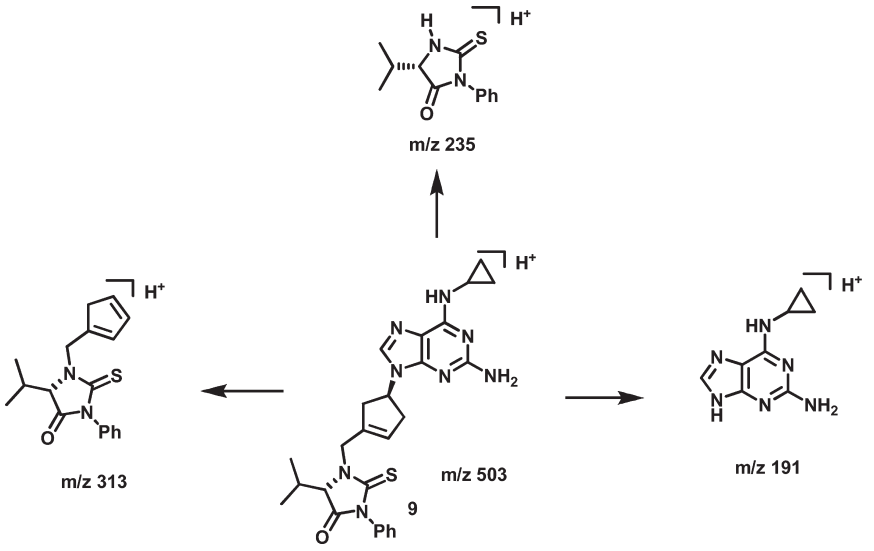
Despite the strong MS evidence for the formation of **8**, any attempts at isolation of this adduct by semipreparative HPLC failed due to coelution with other components of the reaction mixture under a variety of elution conditions. Nonetheless, since valine adducts are usually characterized as hydantoins (e.g., phenylthiohydantoins) upon N-alkyl Edman degradation[23] and our ultimate goal required the appplication of this derivatization strategy in a subsequent step, we elected to characterize **8** as an Edman degradation product, using phenyl isothiocyanate[38] as the derivatizing agent and LC-ESI-MS/MS for adduct characterization. The derivatization resulted in considerable simplification of the HPLC profile (not shown), allowing the isolation of the abacavir-derived phenylthiohydantoin **9** (Scheme 3), by semipreparative HPLC, in 15% yield.

Prior to isolation of adduct **9**, we conducted a series of preliminary experiments to establish the most suitable reaction conditions. HPLC analysis of the reaction mixtures obtained after incubation of aldehyde **5** with ethyl valinate, reduction, and N-alkyl Edman degradation indicated that the efficiency of the procedure decreased with increasing incubation times, which can be explained on the basis of both the reversibility of Schiff base formation and the instability of aldehyde **5** in solution. By contrast, the amount of reducing agent did not affect the isolated yield. Davies et al.[36] reported that use of a large excess of the same reducing agent strongly decreased the yield of the valine monoadduct from 2,5-furandialdehyde due to concomitant reduction of the free aldehyde group. We cannot exclude that any aldehyde still present in our incubation mixtures was also reduced upon addition of the cyanoborohydride; nonetheless, our results suggest that the imino group was reduced very efficiently, which is in line with the extensive use of sodium cyanoborohydride in reductive aminations. Overall, we found the optimized experimental conditions to obtain **8** to consist of a 30-min incubation of **5** with a 3.9-fold molar excess of ethyl valinate at room temperature in 50 mM phosphate (pH 7.4)/THF (0.4:1), followed by the addition of 9−11 M equiv of sodium cyanoborohydride and overnight incubation at room temperature; the ensuing N-alkyl Edman derivatization to **9** was then performed using a standard procedure.[28]

Initial evidence for the formation of adduct **9** was obtained from analysis of the ^{1}H NMR spectrum, which showed all expected resonances from the abacavir and phenylthiohydantoin moieties and a cyclopentene pattern consistent with a C1−C2 double bond (Figure S1, Supporting Information). Conclusive proof of the connection between the two moieties was obtained from the $^{1}H-^{13}C$ 3-bond HMBC correlations (Figure 3) between the bridging geminal protons (two multiplets at 5.14−5.17 ppm and 4.26−4.27 ppm; labeled HydH9 in Figure 3) and both the thiocarbonyl carbon of the hydantoin (183.0 ppm; labeled HydC2) and the olefinic tertiary carbon of the cyclopentene (126.8 ppm; labeled CPC2). Moreover, the same geminal protons both had 2-bond HMBC correlations with the quaternary olefinic carbon of the cyclopentene (137.0 ppm; labeled CPC1). In addition, the ^{13}C NMR signal of the bridging methylene carbon (44.5 ppm; not shown) was relatively shielded when compared with the corresponding carbon in abacavir (the hydroxymethyl carbon; 65.4 ppm), which is entirely consistent with direct connectivity to a nitrogen, rather than the more deshielding oxygen atom. Further evidence for the structure of adduct **9** was obtained by ESI-MS, which showed the protonated molecule at m/z 503. Furthermore, the ESI-MS/MS analysis of MH^+ showed three fragment ions (Scheme 4), stemming from the loss of the purine moiety (m/z 313), loss of the abacavir moiety (m/z 235), and cleavage of the purine−cyclopentene bond, with protonation on the purine moiety (m/z 191), respectively. Taken together, all the spectroscopic data fully support the structure assigned to adduct **9**.

*Reaction of Aldehyde **6** with Ethyl Valinate.* When the β,γ-unsaturated aldehyde **6** was reacted with ethyl valinate under the experimental conditions optimized for the reaction with **5**, the isolated N-alkyl Edman product was again **9**, obtained in 3%

Scheme 4. Proposed ESI-MS/MS Fragmentation Pattern for the Protonated Molecule (m/z 503) of Thiohydantoin 9



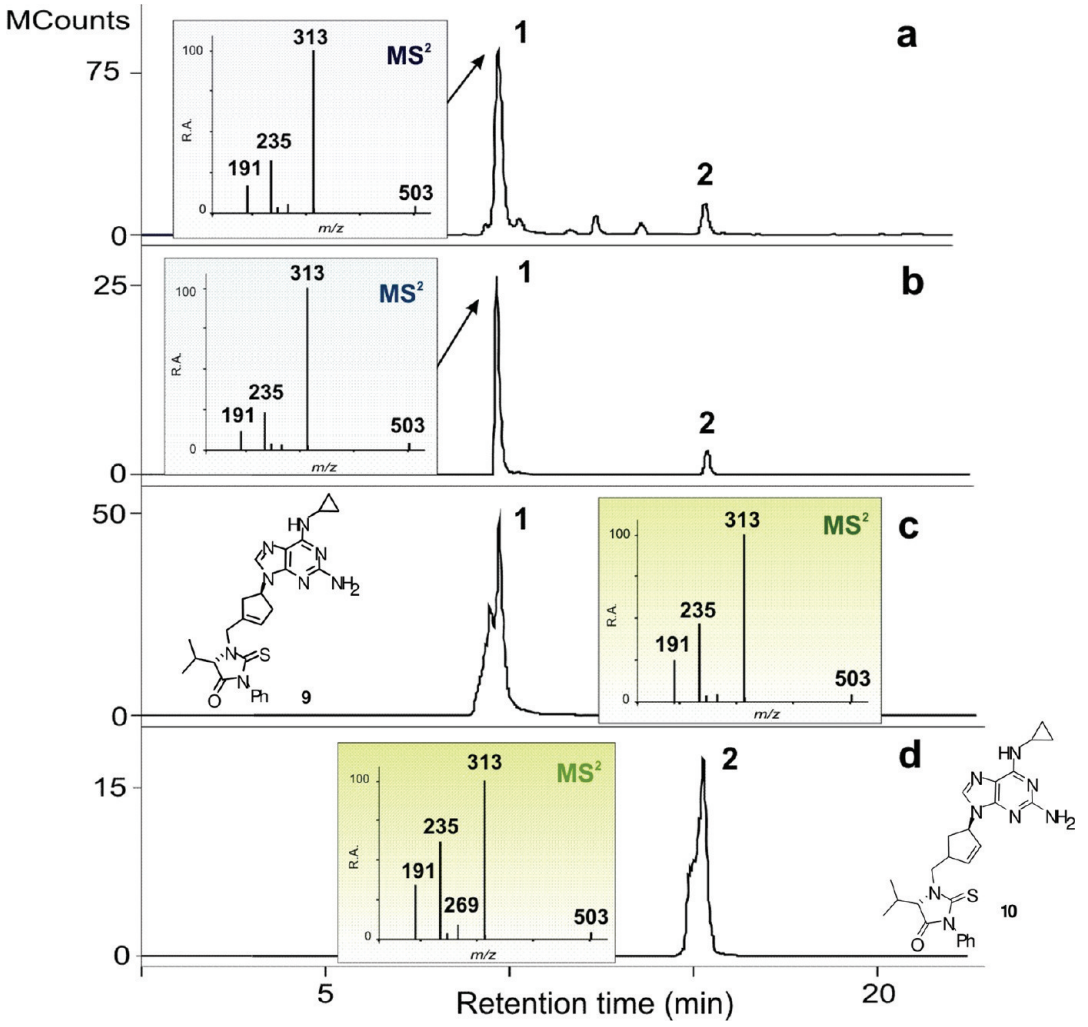


Figure 4. LC-ESI-MS/MS chromatograms of ion m/z 503 obtained after reduction and Edman degradation of (a) the reaction mixture of ethyl valinate modified with **5**; (b) the reaction mixture of ethyl valinate modified with **6**; (c) the synthetic adduct standard **9**; (d) putative synthetic adduct **10**. The elution conditions are outlined in Materials and Methods.

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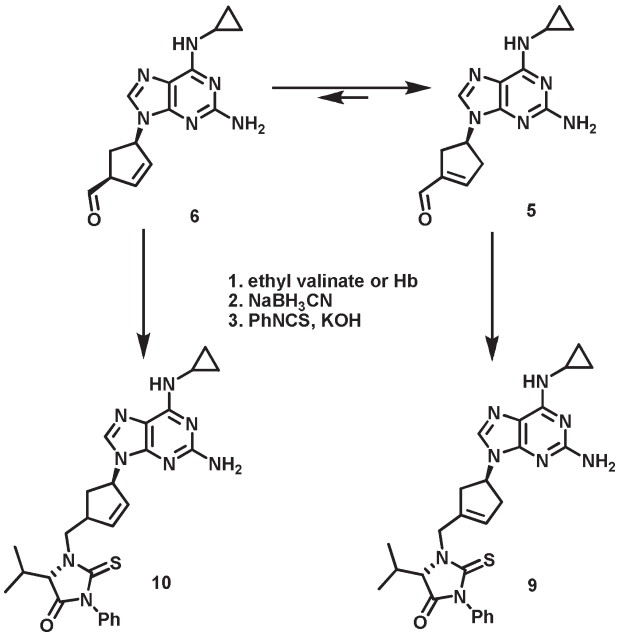
yield. Thus, LC-ESI-MS/MS analysis of the m/z 503 ions provided clear evidence that the major Edman product formed in the reaction mixture from aldehyde **5** (Figure 4, panel **a**, peak 1), characterized as adduct **9**, had the same retention time (ca. 7.5 min) and fragmentation pattern of the major product formed from aldehyde **6** (Figure 4, panel b). As displayed in Figure 4 (panels a and b), a minor signal (peak 2) was observed in both incubation mixtures at higher retention time (ca. 16 min). The protonated molecule corresponding to this signal had m/z 503, identical to adduct **9**; likewise, the MS/MS pattern of this ion was almost identical to the one obtained from adduct **9**, with the exception of a slightly more intense ion at m/z 269, consistent with the loss of the hydantoin moiety (Figure 4, cf. panels c and d). These observations imply that peak 2 corresponded to adduct **10** (Scheme 5), resulting from initial nucleophilic attack by ethyl valinate on the carbonyl of aldehyde **6**. The substantial difference in retention times obtained for the isomeric adducts **9** and **10** under our chromatographic conditions (Figure 4) suggests that the location of the cyclopentene double bond affected the overall molecular geometry and, consequently, the affinity toward the stationary phase.

To obtain further evidence for structure **10**, the fraction corresponding to peak 2 was isolated by semipreparative HPLC and analyzed by ^{1}H NMR. However, only signals from a thiohydantoin originated from direct reaction of ethyl valinate with phenyl isothiocyanate were detected (not shown). The low amount of adduct **10** formed and coelution with this contaminant under the chromatographic conditions used explain our lack of success. Nonetheless, the results obtained by LC-ESI-MS/MS indicate that the β,γ-unsaturated aldehyde **6** rapidly isomerizes to the thermodynamically more stable α,β-unsaturated aldehyde **5**, which is the electrophilic intermediate mainly responsible for reaction with nucleophiles (Scheme 5). Assuming that a similar situation will occur *in vivo*, we propose that aldehyde **5** is the abacavir metabolite primarily involved in covalent adduct formation with biomacromolecules.

Reaction of Aldehyde 5 with Lysine. As discussed above, aldehydes **5** and **6** would be expected to react with side chain and terminal amino groups present in proteins (e.g., lysine and valine residues) via Schiff base formation. We anticipated aldehyde **5** would react also by Michael-type 1,4-addition, based on the results of Walsh et al.[13] and the typical reactivity of α,β-unsaturated aldehydes. For instance, it has long been demontrated that these species can form both Schiff bases and 1,4-addition products with the N-terminal valine of hemoglobin.[39] Likewise, 4-oxo-2-nonenal, a product of lipid peroxidation, selectively modifies the nucleophilic side chains of histidine, lysine, and arginine predominantly by initial Michael addition, although the softer sulfhydryl groups of cysteine residues are kinetically preferred targets.[40,41] However, LC-ESI-MS analyses of the reaction mixtures obtained after incubation of the abacavir aldehydes with ethyl valinate (with or without subsequent reduction) followed by Edman degradation showed no evidence for adduct formation by Michael-type 1,4 addition to the double bond of aldehyde **5** (*vide supra*).

To investigate if other nitrogen nucleophiles would also react exclusively by addition to the carbonyl, we incubated aldehyde **5** with lysine. Although we were unable to obtain sufficient material for NMR analysis, LC-ESI-MS provided clear evidence of adduct formation. As shown in Figure 5, the extracted ion chromatogram (m/z 415) showed two signals with retention times 10.3 and 15.6 min under the chromatographic conditions used and having

Scheme 5. Isomerization of Abacavir Aldehyde 6 to Its α,β-Unsaturated Isomer 5[a]



[a] This isomerization accounts for the predominant formation of adduct **9** in incubations of ethyl valinate or human hemoglobin with any of the two synthetic aldehydes, followed by reduction and *N*-alkyl Edman degradation. Adduct **9** was also the major product identified, upon similar post-modification treatment, in incubations of abacavir with rat liver cytosol.

similar mass spectra. Both displayed protonated (m/z 415) and diprotonated (m/z 208) molecules compatible with adducts stemming from Schiff base formation. Moreover, the presence of a fragment ion at m/z 286 was consistent with cleavage of an N−C bond of lysine, with the nitrogen remaining attached to the abacavir moiety. Lysine has two potential nucleophilic amino groups with relatively close pK_a values [10.5 (side chain) and 9.0 (α-amino group)].[42] Thus, under our experimental conditions (pH 7.4) significant fractions of un-ionized side chain and α-amino nitrogens were available for nucleophilic attack; this explains the formation of two adducts, for which we propose the isomeric structures **11** and **12** (Figure 5c) based upon the MS data. By contrast, the extracted ion chromatogram of m/z 431 (not shown), which would correspond to the protonated molecule of a Michael adduct, did not indicate any component of the mixture with a mass spectral pattern compatible with adduct formation by 1,4-addition. Thus, although we cannot assign a specific structure to each of the adducts formed in the reaction with lysine, our data provide clear evidence that the conjugated aldehyde **5** does not react with lysine via 1,4-Michael type addition.

Reaction of Aldehyde 5 with Cysteine. Walsh et al.[13] observed that the dihydro analogue of abacavir yielded considerably less nonextractable radioactivity than abacavir upon incubation of each of the [^{14}C]-labeled compounds with α,α human ADH isozyme. Assuming that Schiff base formation should not, in principle, be dependent on the presence of the double bond, these data led the authors to propose that 1,4-addition to **5** plays a significant role on the haptenation mechanism of abacavir. Although our results with nitrogen nucleophiles gave no evidence of

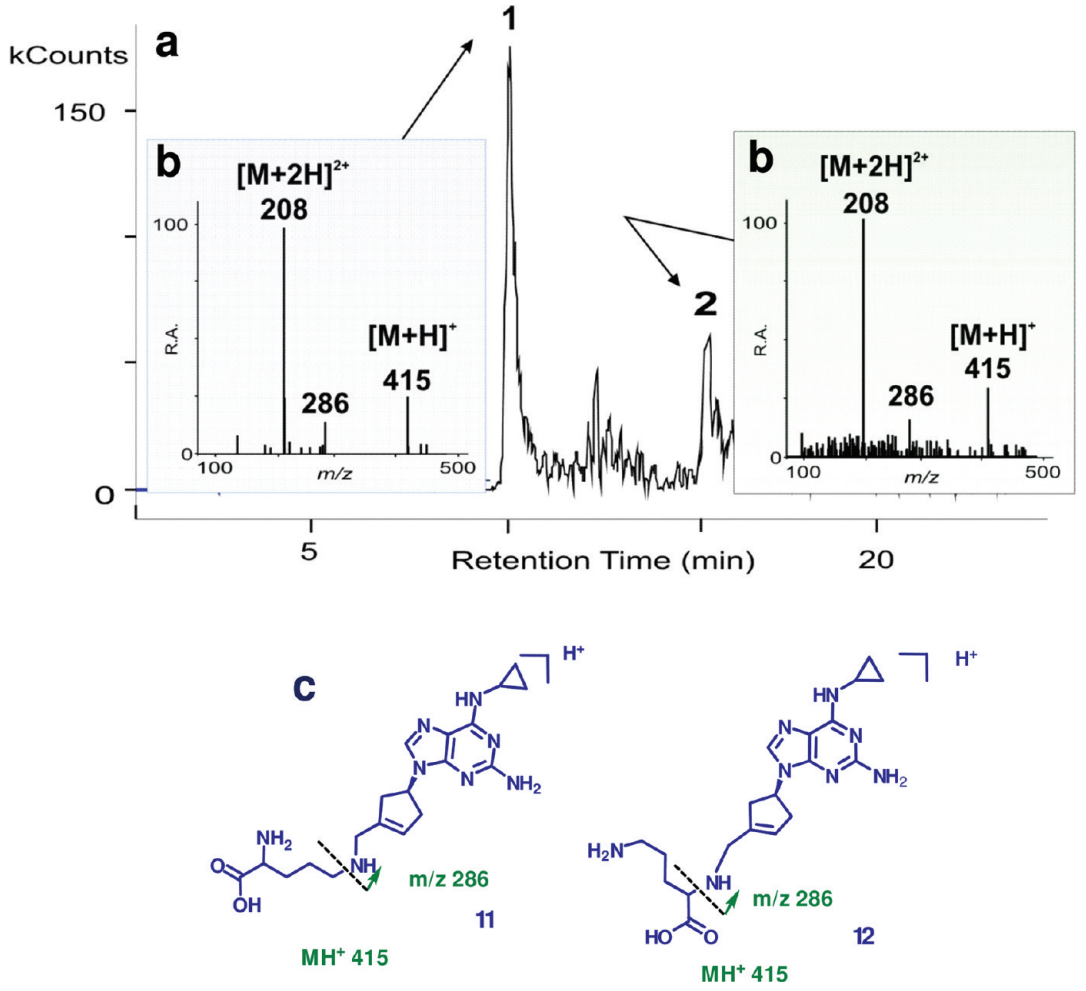


Figure 5. (a) Extracted LC-ESI-MS ion chromatogram (m/z 415) and (b) mass spectra of the protonated adducts, obtained after the incubation of **5** with lysine, followed by treatment with sodium cyanoborohydride; (c) proposed fragmentation mechanism for adducts **11** and **12**. The elution conditions are outlined in Materials and Methods.

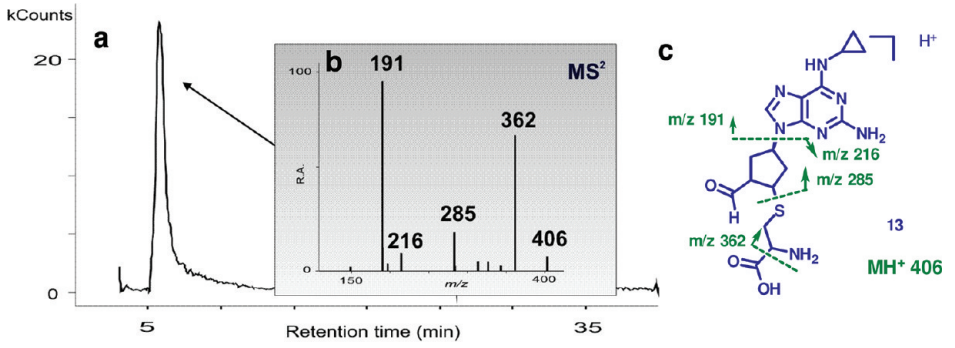


Figure 6. (a) Extracted LC-ESI-MS ion chromatogram (m/z 406) and (b) mass spectrum of the protonated adduct obtained after the incubation of **5** with cysteine; (c) proposed fragmentation mechanism for adduct **13**.

1,4-addition, a different scenario was anticipated for thionucleophiles for two major reasons: (i) contrary to what occurs in the formation of a Schiff base, the initial product of reversible 1,2-addition of a thionucleophile to a carbonyl group cannot be stabilized by dehydration, and (ii) since sulfur is the softest nucleophile in biological systems, it is expected to react readily with an electrophile of similar softness, i.e., the conjugated double bond, rather than the carbonyl group. As a result, cysteinyl residues tend to be the major sites of protein modification by α,β-unsaturated aldehydes.[43,44] To investigate this matter, we incubated aldehyde **5** with cysteine. Although the low amount of product obtained precluded structural analysis by NMR, LC-ESI-MS analysis of the reaction mixture (Figure 6) showed the presence of a signal whose fragmentation pattern was

completely consistent with structure **13**, the adduct stemming from 1,4 Michael-type addition of sulfur to the α,β-usaturated carbonyl **5**.

Incubation of Abacavir with Rat Liver Cytosol. To gain further insight into the isomerization of aldehyde **6** to aldehyde **5** under biologically plausible conditions, we incubated abacavir with rat liver cytosol, reported by Walsh et al.[13] to possess the ADH activity required for metabolic conversion of abacavir into **5** and **6**. The aldehydes thus generated were subsequently trapped with ethyl valinate, and the Schiff bases were stabilized by reduction and derivatized with phenyl isothiocyanate, as described for the synthetic standards. The incubations were performed at pH 8.8, given that significantly more metabolism was observed in cytosol at this pH than at pH 7.4.[13] The incubations were run in triplicate, and aliquots were removed after 2 h and after 20 h. The ethyl acetate extracts obtained after N-alkyl Edman degradation were analyzed by LC-ESI-MS/MS of ion m/z 503, by comparison with the available synthetic standards (Figure 7, panels a−c). We obtained clear evidence for the formation of adduct **9** (panel b), as shown by the extracted ion chromatogram of m/z 313 in an aliquot obtained after 2 h of incubation (panel a); under these conditions, the putative adduct **10** (panel c) was not detected.

Based upon calibration with the synthetic standard, we estimated the concentration of adduct **9** (i.e., after correction for background levels) to be 4.26 ± 1.27 μg/mL in the aliquot analyzed at the 2 h time point. Assuming quantitative derivatization steps and ignoring the formation of the carboxylate metabolite (which was not monitored), this corresponds to ca. 32% conversion of abacavir into the conjugated aldehyde **5** after 2 h, at an average rate of approximately 35 pmol/mg protein/min. We calculated a limit of detection of 0.31 μg/mL for adduct **9** in our LC-ESI-MS/MS conditions, corresponding to ca. 12 pmol on column. Assuming similar ionization responses for adducts **9** and **10**, our failure to detect adduct **10** in the samples from the cytosol incubations implies that, if present, this adduct was formed at less than 10% the level of adduct **9**.

Despite being underestimated, the extent of conversion obtained after a 2 h incubation demonstrates high metabolic capacity (presumably ADH activity) for abacavir oxidation in rat liver cytosol, in agreement with the qualitative data reported by Walsh et al.[13] Moreover, our results provide unequivocal evidence for isomerization of the initial oxidation product, aldehyde **6**, to aldehyde **5** in the cytosol, which supports the proposed role of **5** as the relevant electrophile *in vivo*. Not unexpectedly, the concentration of adduct **9** decreased to background levels (not shown) at the 20 h time point; this decrease can be explained by further metabolism to the abacavir carboxylate **4**[13] and/or side reactions of the aldehyde(s).

Reaction of Aldehyde 5 with Human Hemoglobin. We subsequently investigated the modification of human Hb *in vitro* with the α,β-unsaturated aldehyde **5**. Incubations were conducted at 37 °C for 30 or 60 min; the mixtures were then treated with sodium cyanoborohydride and subjected to N-alkyl Edman degradation, and the ethyl acetate extract was analyzed by LC-ESI-MS/MS of ion m/z 503. By comparison with the preprepared and fully characterized synthetic standards, we confirmed the presence of the thiohydantoin **9** as the major product, but adduct **10** was also detected as a minor product (Figure 7, panel d). Upon quantification, we determined that the 30 min incubation with Hb led to a modification level of 7.0 nmol adduct **9**/mg of protein, which corresponds to the adduction of approximately

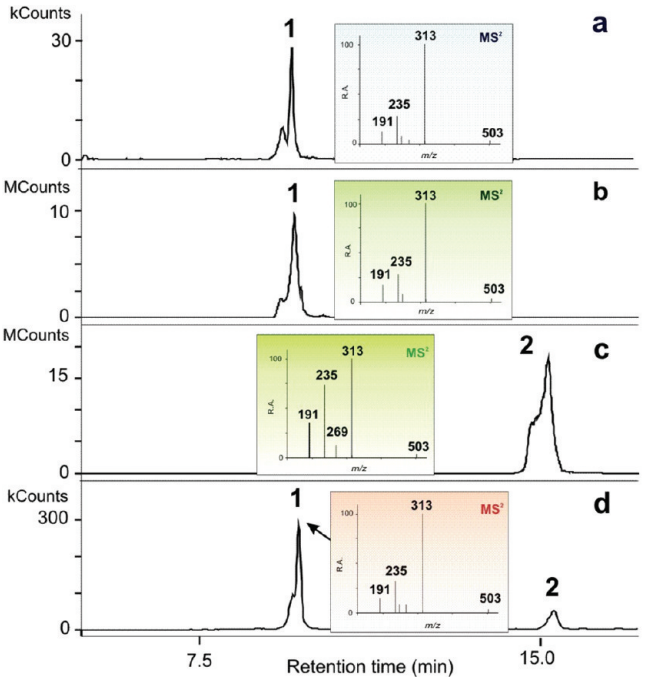


Figure 7. (a) Extracted ion chromatogram (m/z 313) obtained following LC-ESI-MS/MS analysis of ion m/z 503 from a representative reaction mixture obtained after a 2 h incubation of abacavir with rat liver cytosol, followed by trapping with ethyl valinate, reduction, and Edman degradation. LC-ESI-MS/MS chromatogram (m/z 503) of (b) adduct standard **9**; (c) putative synthetic adduct **10**; (d) human Hb modified with **5**, followed by reduction and Edman degradation. The elution conditions are outlined in Materials and Methods.

45% of the N-terminal valines. As observed for the incubations with rat liver cytosol, the longer incubation time (60 min in this case) resulted in a substantial decrease of the modification level (to ca. 3.9 nmol adduct **9**/mg of protein).

These results are a clear indication of reaction between aldehyde **5** and the N-terminal valine of Hb. Moreover, the detection of adduct **10**, formed from the β,γ-unsaturated aldehyde **6**, suggests that, regardless of the starting aldehyde (**5** or **6**), an equilibrium between the two species is established in solution.

With the aim of characterizing the abacavir-modified amino acids in human Hb, the mixtures from additional incubations with aldehyde **5** were subjected to enzymatic hydrolysis with Pronase E and leucine aminopeptidase M (with and without prior reduction with sodium cyanoborohydride), and the hydrolysates were analyzed by LC-ESI-MS/MS. We searched for specific adducts, with particular emphasis on ions m/z 415 (the protonated lysine adduct), m/z 406 (the protonated cysteine adduct), and m/z 408 (the protonated cysteine adduct after reduction of the carbonyl group). However, we were unable to detect any abacavir-derived adducts in the enzymatic hydrolysates. In a previous study, we have successfully used this methodology to characterize nevirapine-amino acid adducts formed in human Hb and serum albumin *in vitro*.[29] Considering our ready detection of the N-terminal valine adduct following Edman degradation, it is implausible that no reaction occurred at other nucleophilic sites in the protein. The failure to detect abacavir-Hb adducts in the enzymatic hydrolysates may have been due to the inherent instability of the adducts under the hydrolysis conditions. In addition, we cannot exclude the fact

that initially formed Michael adducts (e.g., at cysteinyl residues) underwent subsequent Schiff base formation through the carbonyl moiety, producing cross-linked products.[45,46] Although we obtained a ready reaction of aldehyde **5** with the un-ionized cysteine thiol at the monomer level, the fact that human hemoglobin is reported to lack a highly reactive cysteine (i.e., as a thiolate)[47] may have also been a factor. Nonetheless, it should be noted that we also modified human serum albumin with aldehyde **5** but were unable to identify any adducts in the enzymatic hydrolysate, despite the fact that human serum albumin contains one cysteine (C34) in the highly nucleophilic thiolate form due to the vicinity of three ionizable residues, D38, H39, and Y84, within the tertiary structure of the protein.[48] As such, the N-terminal valine residue in Hb appears as the more convenient surrogate to probe abacavir activation to a reactive electrophile.

CONCLUSIONS

To gain insight into the role of aldehyde metabolites in abacavir-induced toxicity, we synthesized the two putative abacavir aldehyde intermediates, one conjugated (**5**) and the other nonconjugated (**6**). We further demonstrated that both aldehydes react with nitrogen bionucleophiles to yield the same adduct. Specifically, reaction of the conjugated aldehyde **5** with ethyl valinate, followed by reduction and N-alkyl Edman degradation, led to a thiohydantoin (**9**) that was also formed as the major product from reaction of aldehyde **6** with ethyl valinate under similar experimental conditions. Likewise, adduct **9** was the only adduct detected by LC-ESI-MS/MS analysis of the ethyl acetate extract obtained from rat liver cytosol upon trapping the two ADH-generated aldehydes with ethyl valinate, followed by the same reduction and Edman degradation procedures. These results suggest that, once formed *in vivo*, aldehyde **6** will isomerize to the thermodynamically more stable conjugated aldehyde **5**, which is expected to be the electrophilic abacavir metabolite primarily responsible for haptenation to proteins. Moreover, given the considerably high yield (ca. 32%) obtained for adduct **9** in the cytosol incubations, the formation of aldehyde **5** should be regarded as a major metabolic pathway for abacavir.

Metabolite **5** consistently reacted with nitrogen (hard) bionucleophiles through Schiff base formation. By contrast, the adduct detected by LC-ESI-MS/MS analysis of the mixture obtained from reaction with the soft sulfur nucleophile cysteine was the expected product of Michael-type addition to the α,β-unsaturated system. These observations reflect a nucleophile-specific regioselectivity for the addition to **5**, which could also occur *in vivo*.

Modification of Hb *in vitro* with aldehyde **5**, followed by reduction, N-alkyl Edman degradation, and LC-ESI-MS/MS analysis allowed the ready detection and unequivocal identification of adduct **9** in significant yield. By contrast, no products of Michael-type addition were identified from reaction of aldehyde **5** with human serum albumin, despite the fact that this protein contains a highly reactive cysteine thiolate. Regardless of the potential toxicological relevance of other unidentified abacavir-derived adducts, the availability of a reliable, fully characterized, synthetic standard of **9**, and its easy detection in the model Hb modifications, anticipate the usefulness of this adduct as a prospective biomarker of abacavir toxicity in humans. We are currently conducting *in vivo* studies to test this hypothesis.

ASSOCIATED CONTENT

Supporting Information. ^{1}H NMR spectrum of adduct **9**, recorded in methanol-d_4. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

ADH, alcohol dehydrogenase; ALDH, aldeyde dehydrogenase; DMF, *N*,*N*-dimethylformamide; DMSO, dimethylsulfoxide; ESI, electrospray ionization; Hb, hemoglobin; HIV, human immunodeficiency virus type 1; HSR, hypersensitivity reaction; LC, liquid chromatography; MS/MS, tandem mass spectrometry; THF, tetrahydrofuran

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