Structure-Based Design of HIV Protease Inhibitors: 5,6-Dihydro-4-hydroxy-2-pyrones as Effective, Nonpeptidic Inhibitors

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From a broad screening program, the 4-hydroxycoumarin phenprocoumon (**I**) was previously identified as a lead template with HIV protease inhibitory activity. The crystal structure of phenprocoumon/HIV protease complex initiated a structure-based design effort that initially identified the 4-hydroxy-2-pyrone U-96988 (**II**) as a first-generation clinical candidate for the potential treatment of HIV infection. Based upon the crystal structure of the 4-hydroxy-2-pyrone **III**/HIV protease complex, a series of analogues incorporating a 5,6-dihydro-4-hydroxy-2-pyrone template were studied. It was recognized that in addition to having the required pharmacophore (the 4-hydroxy group with hydrogen-bonding interaction with the two catalytic aspartic acid residues and the lactone moiety replacing the ubiquitous water molecule in the active site), these 5,6-dihydro-4-hydroxy-2-pyrones incorporated side chains at the C-6 position that appropriately extended into the S_1' and S_2' subsites of the enzyme active site. The crystal structures of a number of representative 5,6-dihydro-4-hydroxy-2-pyrones complexed with the HIV protease were also determined to provide better understanding of the interaction between the enzyme and these inhibitors to aid the structure-based drug design effort. The crystal structures of the ligands in the enzyme active site did not always agree with the conformations expected from experience with previous pyrone inhibitors. This is likely due to the increased flexibility of the dihydropyrone ring. From this study, compound **XIX** exhibited reasonably high enzyme inhibitory activity (K_i = 15 nM) and showed antiviral activity (IC_{50} = 5 μM) in the cell-culture assay. This result provided a research direction which led to the discovery of active 5,6-dihydro-4-hydroxy-2-pyrones as potential agents for the treatment of HIV infection.

Introduction

The rapid spread of the acquired immune deficiency syndrome (AIDS) epidemic has stimulated discovery for therapeutic agents to arrest the replication of the causative virus, human immunodeficiency virus (HIV). One promising possibility to interrupt the viral life cycle is the use of inhibitors of the virally encoded protease which is indispensable for viral maturation.[1,2] Among the most potent inhibitors reported thus far are peptidomimetic compounds containing transition-state inserts in place of the dipeptidic cleavage sites of the substrates.[3−6] The low oral bioavailability and rapid biliary excretion of peptide-derived compounds[7] have limited their utility as potential therapeutic agents. Recent advances have resulted in HIV protease inhibitors with reduced peptidic character and also nonpeptidic inhibitors that are more orally bioavailable, and an increasing number of HIV protease inhibitors[8−21] are currently undergoing clinical evaluations.

In a recent communication,[20] we reported the identification of phenprocoumon (3-(α-ethylbenzyl)-4-hydroxycoumarin, compound **I** in Figure 1, K_i = 1 μM) from a broad screening program as an active HIV protease inhibitory template. It is noted that other independent studies[22−26] have also described 4-hydroxybenzopyran-2-ones and 4-hydroxypyran-2-ones as inhibitors of HIV protease. The increasing number of reported crystal structures of inhibitor/HIV protease complexes have provided numerous successful examples of structure-based designs of potent HIV protease inhibitors.[27,28] For our research program, the crystal structure of inhibitor **I**/HIV-1 protease complex formed the basis of iterative cycles of structure-based design of more active analogues. From that investigation,[20] we studied a class of compounds in the 4-hydroxy-2-pyrone series, and U-96988 (3-(α-ethylbenzyl)-6-(α-ethylphenethyl)-4-hydroxy-2*H*-pyran-2-one, compound **II** in Figure 1, K_i = 38 nM) was identified as the first clinical

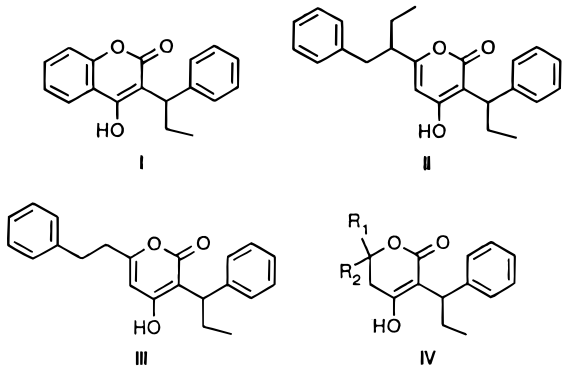

Figure 1. Structures of HIV protease inhibitors.

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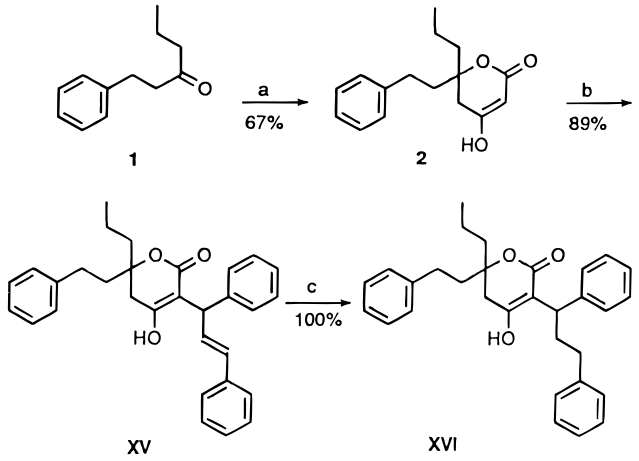
Scheme 1. Synthetic Route via a Lewis Acid-Catalyzed Condensation Reaction[a]



[a] (a) $CH_3COCH_2CO_2Me$, NaH, *n*-BuLi, THF; (b) $C_6H_5CH{=}CH$-$CH(OH)C_6H_5$, BF_3-Et_2O, dioxane; (c) 10% Pd/C, H_2(g), EtOAc.

candidate in this promising series of nonpeptidic HIV protease inhibitors as a potential therapeutic agent for the treatment of HIV infection. In that report,[20] we described the crystal structure of the HIV-1 protease complex with the 4-hydroxy-2-pyrone inhibitor **III** (K_i = 0.5 μM). We noted that the phenethyl group at C-6 of the inhibitor **III** (see Figure 2, top left) was not able to occupy the S_1' and S_2' pockets in the enzyme active site. It was postulated that a corresponding 5,6-dihydro-4-hydroxy-2-pyrone inhibitor such as compound **IV** might allow the two substituents at C-6, with the sp^3 hybridization, to extend into the S_1' and S_2' subsites (see Figure 2, top left). This report describes our initial structure−activity study of a series of HIV protease inhibitors in the 5,6-dihydro-4-hydroxy-2-pyrone template, which has also been independently studied.[29]

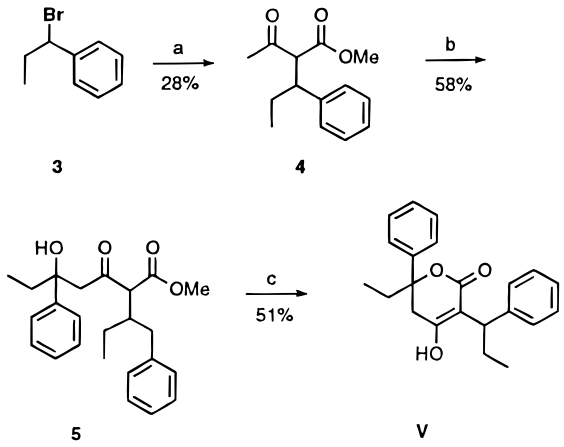
Chemistry

Compounds **XV** and **XVI** were prepared via a Lewis acid-catalyzed condensation procedure as shown in Scheme 1. Addition of the dianion of methyl acetoacetate to phenethyl propyl ketone (**1**) was followed by a ring closure after workup to provide the dihydropyrone **2**. Condensation of compound **2** with 1,3-diphenyl-2-propen-1-ol using boron trifluoride etherate as a catalyst gave compound **XV**. A highly stabilized carbonium ion intermediate seemed to be required for this condensation reaction to be successful since an α-alkylbenzyl alcohol was not useful in this reaction. Hydrogenation of the olefin functionality in compound **XV** then resulted in the corresponding saturated analogue **XVI**.

Since compound **V** could not be prepared by the Lewis acid-catalyzed condensation procedure of a dihydropyrone ring using α-ethylbenzyl alcohol, it was prepared via an alkylation procedure as shown in Scheme 2. Alkylation of the dianion of methyl acetoacetate with α-ethylbenzyl bromide (**3**) gave the intermediate **4**. The corresponding dianion of compound **4** was then added to propiophenone to give the aldol adduct **5**. Hydrolysis of the methyl ester was followed by a spontaneous ring closure to give the desired dihydropyrone **V**.

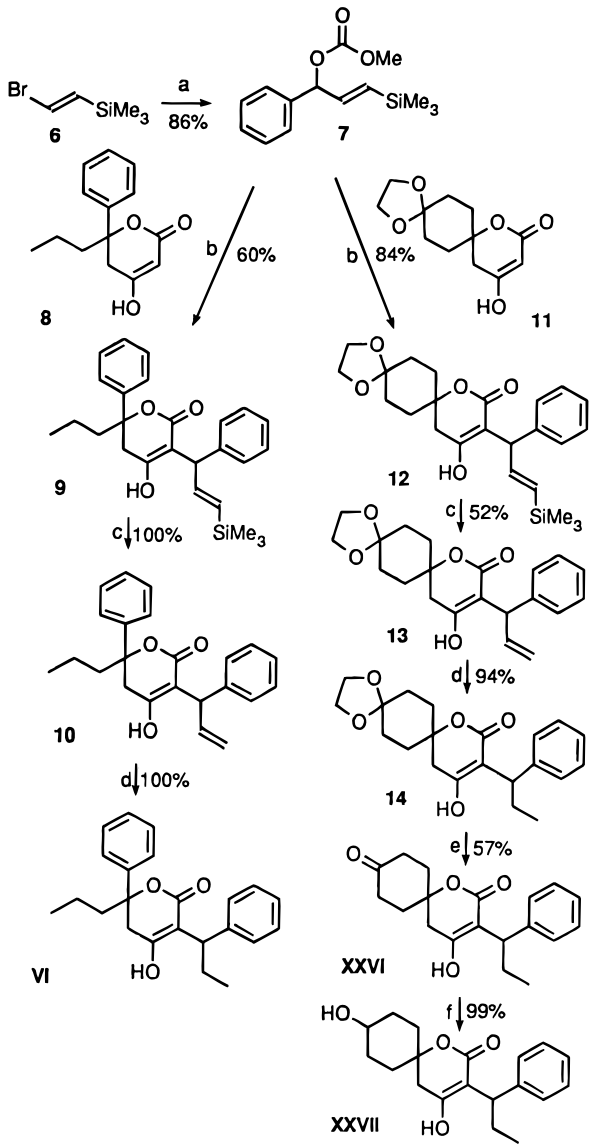
A more efficient route for the preparation of a dihydropyrone with a C-3 α-ethylbenzyl substituent was devised as shown in Scheme 3. A palladium-catalyzed allylic alkylation of the dihydropyrone ring was envisaged to install the substitution at C-3. This reaction

Scheme 2. Synthetic Route via an Alkylation Reaction[a]



[a] (a) $CH_3COCH_2CO_2Me$, NaH, THF; (b) NaH, *n*-BuLi, THF, $C_6H_5COC_2H_5$; (c) NaOH, THF/H_2O.

Scheme 3. Synthetic Route via a Palladium-Catalyzed Allylic Alkylation Reaction[a]



[a] (a) *t*-BuLi, C_6H_5CHO, THF, $ClCO_2Me$; (b) $Pd(OAc)_2$, $(C_6H_5)_3P$, $C_6H_5CH_3$, 70 °C; (c) $CH_3C_6H_4SO_3H$, CH_3CN, reflux; (d) 10% $Pd(OH)_2$/C, H_2(g), EtOH; (e) H_3O^+; (f) $NaBH_4$.

for stabilized enolate anions such as malonate is a well-known reaction[30] and has been applied to nucleophiles such as 4-hydroxycoumarin, 4-hydroxy-2-pyrone, and

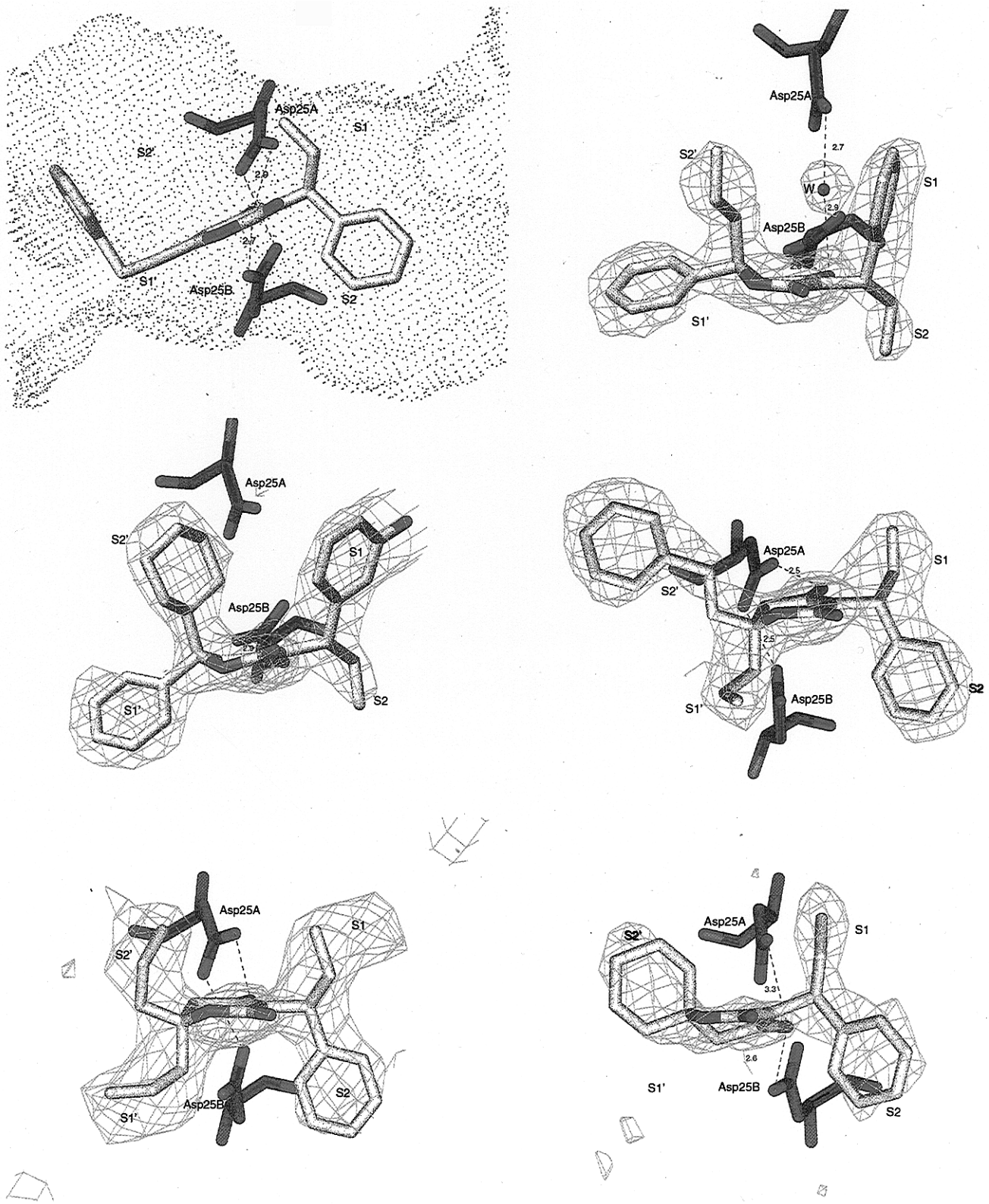
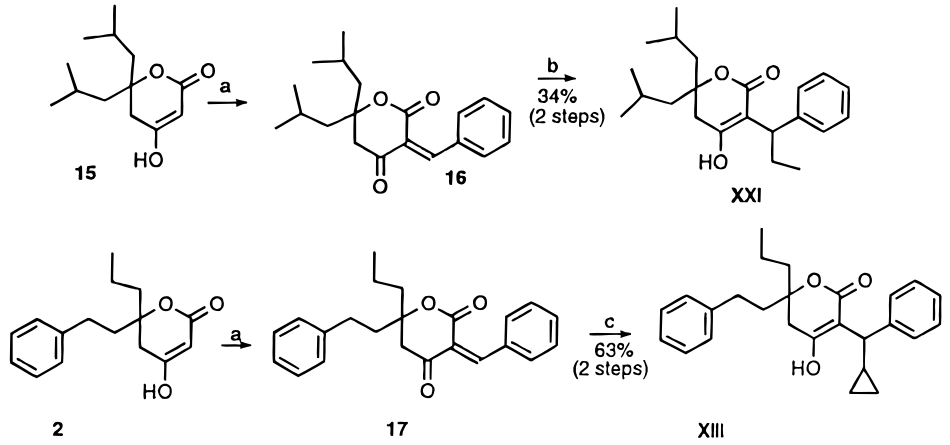


Figure 2. (Top left) X-ray crystal structure of compound **III** bound to HIV-1 protease. The surface of the enzyme is shown with green dots. The carbons of the catalytic aspartic acids are shown in green and the carbons of the inhibitor molecule in white. The pyrone ring of the inhibitor is positioned approximately equidistant from the two aspartic acids. Subsites S2−S2′ are indicated. (Top right) X-ray crystal structure of compound **VI** bound to HIV-2 protease. The hydroxyl group of the inhibitor forms a hydrogen bond to only one of the catalytic aspartic acids with a water-bridged interaction to the other. The phenyl ring attached to the C-3α position is located in the S1 subsite. The inhibitor-removed difference electron density map is shown at the 2s contour level in light blue. (Middle left) X-ray crystal structure of compound **VII** bound to HIV-2 protease. The orientation of this inhibitor is similar to the orientation found in the complex of compound **VI** but without the water molecule or one with very low occupancy. The inhibitor-removed difference electron density map is shown at the 2s contour level in light blue. (Middle right) X-ray crystal structure of compound **XII** bound to HIV-1 protease. The interaction of the hydroxyl group of the inhibitor forms a hydrogen bond to the opposite catalytic aspartic acid when compared to the complex with **VI** or **VII**. The dihydropyrone ring is puckered in the opposite direction, as well. The phenyl ring attached to the C-3α position is located in the S2 subsite. The inhibitor-removed difference electron density map is shown at the 2s contour level in light blue. (Bottom left) X-ray crystal structure of

Scheme 4. Synthetic Route via a 1,4-Conjugated Addition Reaction[a]



[a] (a) C_6H_5CHO, $AlCl_3THF$; (b) Et_3Al, $CuBr-Me_2S$, THF; (c) *c*-C_3H_5MgBr, $CuBr-Me_2S$, THF.

tetronic acid.[31] However, such nucleophiles attack the π-allyl intermediate derived from the 1-phenyl-1-propenol at the least substituted position. For our purpose, we would require the attack at the more substituted position to lead to the desired regioisomer. The solution to this regiochemical control was reported[32] by employing a silyl-substituted allylic carbonate such as compound **7**. The desired reagent could be efficiently prepared via lithium−halogen exchange of commercially available (2-bromovinyl)trimethylsilane (**6**) with *tert*-butyllithium followed by the addition of benzaldehyde to give the intermediate allylic alcoholate. Addition of methyl chloroformate to the reaction mixture resulted in the isolation of the allylic carbonate **7**. Heating of the dihydropyrone **8**, which was prepared in a similar manner to the dihydropyrone **2**, and the allylic carbonate **7** with palladium acetate and triphenylphosphine in toluene gave the desired product **9**. The trimethylsilyl group was removed upon treatment with *p*-toluenesulfonic acid in refluxing acetonitrile to give the olefin **10**. Subsequent hydrogenation afforded the desired saturated analogue **VI**. Compounds **VII**−**XII** were also prepared by this palladium-catalyzed allylic alkylation procedure.

Spiro compounds **XXII**−**XXIX** were also prepared by the palladium-catalyzed allylic alkylation procedure as shown in Scheme 3. The dihydropyrone **11** was heated with the allylic carbonate **7** in the presence of palladium acetate and triphenylphosphine to give the vinylsilane **12**. Treatment with *p*-toluenesulfonic acid removed the trimethylsilyl group to give the olefin **13**, which was reduced by hydrogenation to the saturated analogue **14**. Acidic hydrolysis of the ketal-protecting group resulted in the ketone **XXVI**. Simple reduction of the ketone functionality gave the desired alcohol **XXVII**.

A more versatile procedure to install the C-3 α-substituted benzyl group on the dihydropyrone ring is shown in Scheme 4. The dihydropyrone **15**, which was prepared in a similar manner to the dihydropyrone **2**, was condensed with benzaldehyde using aluminum trichloride as an acidic catalyst to give the isolable intermediate **16**. For the initial preparation of the α-ethyl-containing compound, treatment with triethylaluminum and copper(II) bromide successfully resulted in the desired 1,4-adduct **XXI**. To increase the range of the α-substitution, a copper-catalyzed Grignard addition reaction was explored as shown in Scheme 4. Thus, the dihydropyrone **2** was condensed with benzaldehyde using aluminum trichloride as an acidic catalyst to give the isolable intermediate **17**, as above. Subsequent treatment with cyclopropylmagnesium bromide and copper(II) bromide gave the desired adduct **XIII**. Compounds **XIV** and **XVII**−**XX** were also prepared by this 1,4-addition reaction sequence.

Results and Discussion

With the notion that a corresponding 5,6-dihydro-4-hydroxy-2-pyrone inhibitor such as compound **IV** might allow the two substituents at C-6, with the sp^3 hybridization, to extend into the S_1' and S_2' subsites, some representative compounds with varying substitutions at the C-6 were explored. In the first series of the 5,6-dihydro-4-hydroxy-2-pyrones, a phenyl group was chosen as one of the two substituents at C-6 (compounds **V**−**VIII** in Table 1). The other substituent in each of these compounds was the ethyl, propyl, cyclohexyl, or benzyl group, respectively. The compounds with the relatively large C-6 substituents (compounds **VII** and **VIII**) showed significantly poorer enzyme inhibitory activity as compared to compounds wherein one of the substituents was a small alkyl side chain (compounds **V** and **VI**). When the benzyl group was used as one of the two substituents at C-6 (compounds **VIII**−**X** in Table 1), the resulting compounds showed relatively weak enzyme inhibitory activity even in the case of compound **X** where the second substituent at C-6 was a small alkyl side chain.

These inhibitors were generally found to be equally effective against HIV-1 and HIV-2 proteases. Crystallization of some of these inhibitors with both HIV-1 and HIV-2 proteases was attempted for structural chemistry studies, and only crystals of the HIV-2 protease com-

compound **XVII** bound to HIV-1 protease. The inhibitor is roughly centered in the catalytic site. Only one orientation of the 2-fold overlapped inhibitor molecules is shown. The inhibitor-removed difference electron density map is shown at the 2s contour level in light blue. (Bottom right) X-ray crystal structure of compound **XXIII** bound to HIV-2 protease. The pucker of the dihydropyrone ring is similar to that shown in the top right and middle left structures, but the groups attached to the C-3α position are in the subsites shown in the middle right and bottom left structures. The inhibitor-removed difference electron density map is shown at the 2s contour level in light blue.

Table 1. HIV Protease Inhibitory Activity of Compounds with a Phenyl Group at C-6 and Compounds with a Benzyl Group at C-6

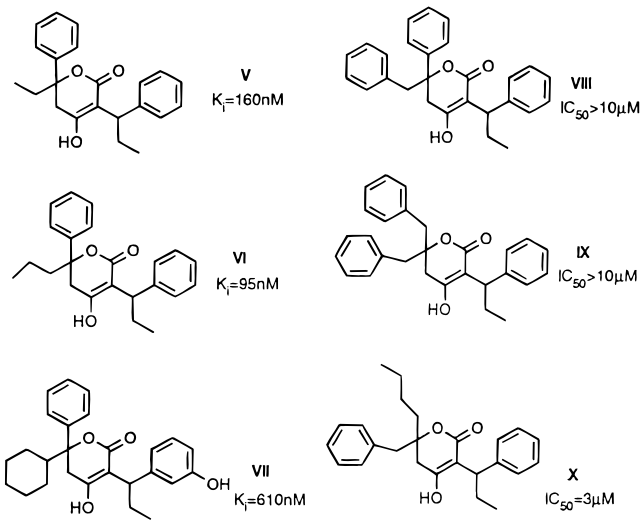
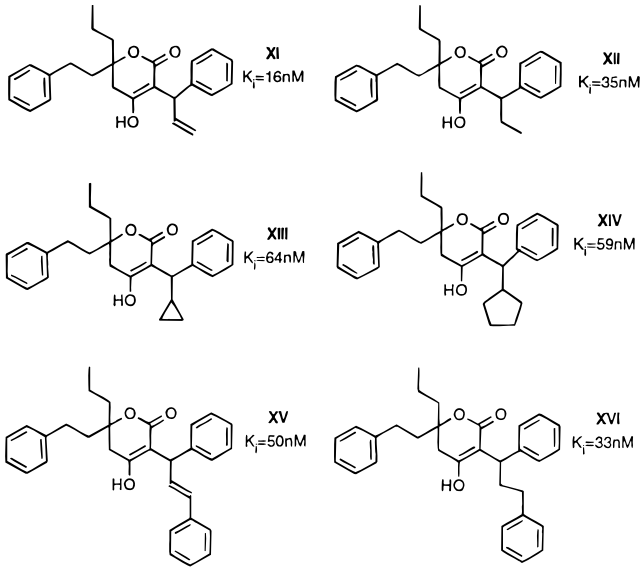


Table 2. HIV Protease Inhibitory Activity of Compounds with a Phenethyl Group at C-6



plexed with compounds **VI** and **VII** were suitable for structural determination. Although HIV-1 and HIV-2 proteases have high homology, subtle differences exist and the level of detailed interpretation of crystal data structures pointing to inhibitor/enzyme interaction can be limited. Discussion of differences in conformations of inhibitors in the enzyme active site in this report needs, therefore, to be understood in the light of this limitation. In general, the crystal structures of this group of compounds show variations in the binding mode of the ligands in the enzyme active site when compared to the previously described 4-hydroxycoumarin and 4-hydroxy-2-pyrone classes of compounds.[20,21] In those structures with a phenyl group and a small alkyl group on the C-3α carbon, the alkyl group was found in the S_1 subsite and the phenyl group in the S_2 subsite. Additionally, the 4-hydroxy group was positioned approximately equidistant between and making hydrogen bonds to the two catalytic aspartic acid residues, and the lactone oxygen atoms replaced the ubiquitous water molecule found in the complexes of the peptidomimetic inhibitors.[27,28] In many previously determined structures of the protease/inhibitor complexes, inhibitors had been observed to bind in two alternate orientations related by the approximate molecular 2-fold axis that bisects the catalytic site. However, in the crystal structures of HIV-2 protease complexed with the inhibitors **VI** (K_i = 28 nM against HIV-2 protease) and **VII**, only one of the possible diastereomers of these inhibitors (3α*S*,6*S* diastereomer of **VI** and 3α*S*,6*R* diastereomer of **VII**) was found, and they bound in only one orientation. The C-3α substituents in **VI** and **VII** were also oriented in directions opposite to that described above for the inhibitors with pyrone and coumarin templates. In addition an unexpected binding in the enzyme active site was found (Figure 2, top right and middle left). The inhibitors were seen to bind asymmetrically, with their 4-hydroxyl groups binding directly to one of the catalytic aspartic acid residues of the enzyme, leading the ring puckering in the direction of the other aspartic acid. The difference electron density map shown in Figure 2, top right, illustrates these features. The two substituents at C-6 of the inhibitors **VI** and **VII** could be found in the respective S_1' and S_2' subsites of the enzyme active site. In the case of **VII** we could have equally chosen to model the diastereomer with 3α*S*,6*S* stereochemistry, with the same direction for the dihydropyrone ring puckering.

It is interesting to note that, in either inhibitor, the carbonyl oxygen of the template ring had nearly symmetrical hydrogen-bonding interactions with the NH groups of residues Ile50A and Ile50B on the flaps of the enzyme. A water molecule located off the second aspartic group that formed a hydrogen bond to the C-4 hydroxyl group in the complex of **VI**, however, did not have its counterpart in the complex of **VII** perhaps due to an additional bulkier group (attached to the C-6 position) crowding the S_2' subsite. The poorer binding of **VII** (relative to **VI**) may, therefore, be the result of bulkier groups located on the C-6 position. The *m*-hydroxyl group on the C-3α phenyl group in **VII** formed weak hydrogen-bonding interactions with the carbonyl oxygen of the residue Gly27A and a discrete water molecule.

The structure−activity relationship results in Table 1 gave support to the notion that a 5,6-dihydro-4-hydroxy-2-pyrone template, which is a structure-based design hypothesis, might allow the two substituents at C-6 to extend into the S_1' and S_2' subsites. This was also confirmed by the crystallographic data of enzyme/inhibitor complexes. As discussed above, the crystallographic data pointed to subtle changes in unexpected consequence of some different binding modes of new inhibitors. As for the structure−activity relationship study in Table 1, a phenyl group at C-6 resulted in compounds with some improvement in potency over the 4-hydroxycoumarin **I** or the 4-hydroxy-2-pyrone **III**. A benzyl group at C-6, however, led to compounds with weaker binding affinity.

With the intent to improve binding affinity, the 5,6-dihydro-4-hydroxy-2-pyrones in Table 2 were suggested and prepared. They all contained a phenethyl group and a propyl group at C-6. The phenethyl group was evaluated since this substituent at C-6 was previously found to be effective for the 4-hydroxy-2-pyrone inhibitor **III**, and the propyl group was chosen since it represented the effective small substituent at C-6 in compound **VI**. All of the 5,6-dihydro-4-hydroxy-2-pyrones in Table 2 also contained a phenyl group at the C-3α

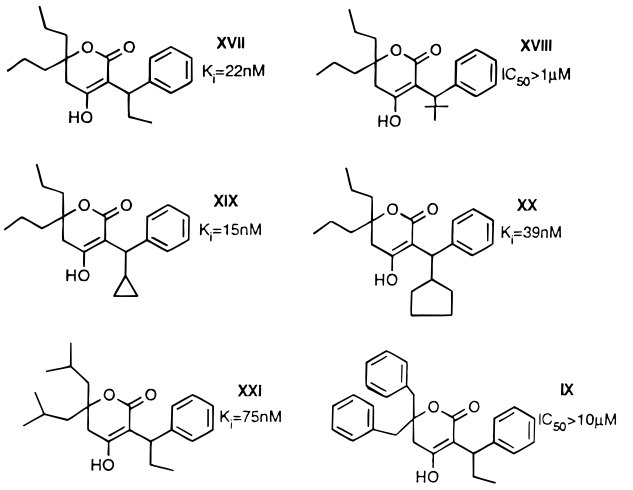
carbon, which has also proven useful in previous series. These six compounds differed in the second substitution at the C-3α position. Although smaller C-3α groups resulted in inhibitors (compounds **XI** and **XII**) with good inhibitory activity (K_i = 16 and 35 nM, respectively), the small cycloalkyl groups (compounds **XIII** and **XIV**) and even larger groups (compounds **XV** and **XVI**) also led to compounds with comparable inhibitory activity (K_i = 30−60 nM), suggesting the lack of stringent requirement for this substituent.

The crystal of the autolysis resistant triple mutant (Q7K/L33I/L63I) of HIV-1 protease[34] complexed with the inhibitor **XII** turned out to be suitable for structural determination (Figure 2, middle right). As with inhibitors **VI** and **VII**, an asymmetrical binding for inhibitor **XII** was noted but with some differences. The dihydropyrone ring in this case was bound to the opposite catalytic aspartic acid group when compared to that found in the complexes of inhibitors **VI** and **VII**. This led to the ring puckering in the opposite direction relative to that in the complexes with inhibitors **VI** and **VII** (compare top right and middle right structures in Figure 2). In other words, the direction of the pucker, when asymmetric binding takes place, would be expected to be correlated with the direction of binding of the 4-OH group of the dihydropyrone ring to one of the two catalytic aspartic acid residues. This off-centered asymmetric binding once again required us to model only one of the four diastereomers, namely, the one with 3α*R*,6*S* stereochemistry, into the difference electron density map illustrated in Figure 2, middle right. The differences noted above, in the binding features exhibited by inhibitors **XII** and **VI** or **VII**, may be minimally dependent upon the six differences (namely, V32I, I47V, and V82I for each monomer) between HIV-1 (wildtype or the triple mutant) and HIV-2 proteases at the S_2'−S_2 subsites. The mutation points in the triple mutant are distal from the active site cleft and have been shown to exert no observable change to the kinetic properties of the protease.[34] The only significant difference that we have noted so far is the way these proteases pack to form crystals: Whereas, the wildtype HIV-1 protease is known to crystallize in five different space groups, the triple mutant has given us crystals only in the $P2_12_12$ space group.

The structure−activity observation made from the compounds in Table 2 was that the specific choices of a phenethyl group and a propyl group at C-6 did result in compounds with improved binding affinity over those in Table 1. Compounds in this new template, with more than 10-fold improvement in K_i values over the reference 4-hydroxy-2-pyrone **III** and having comparable potency to the first clinical candidate **II**, were realized. Again, the crystallographic data suggested subtle changes in the position of inhibitor **XII** in the enzyme active site, as compared to previous observations made with other inhibitors.

Interest in the possibility of removing a chiral center at the C-6 position of the 5,6-dihydro-4-hydroxy-2-pyrone template, which would reduce complexity of diastereomeric mixtures, led us to study a series of compounds with two identical substituents at C-6 (see Table 3) with the intent to maintain comparable enzyme inhibitory potency as found with compounds in Table 2. Compounds **XVII**−**XX** contained two propyl groups at C-6 and a phenyl group at the C-3α carbon and

Table 3. HIV Protease Inhibitory Activity of Compounds with Two Identical Substituents at C-6

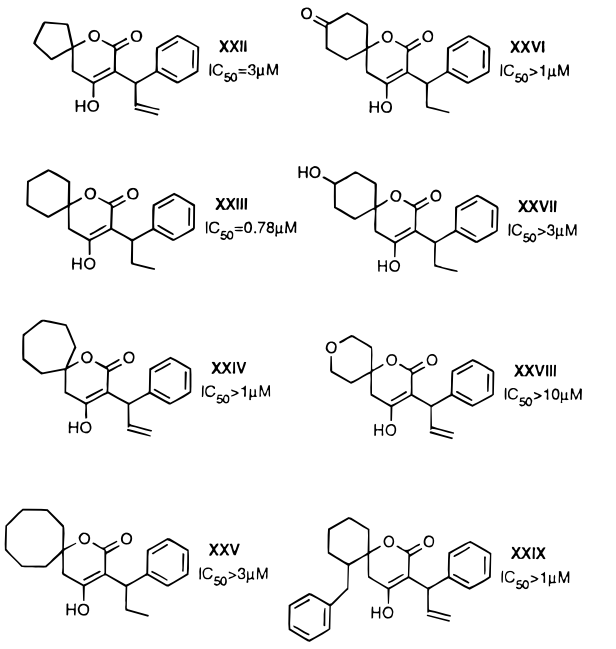


differed in the substitution at the C-3α position. Compound **XVII**, with an ethyl group at the C-3α carbon, showed good inhibitory activity, while the analogue with a *tert*-butyl group, compound **XVIII**, exhibited significantly poorer inhibitory activity, suggesting that the large *tert*-butyl group could not be properly accommodated in the active site pocket. Compounds **XIX** and **XX** contained small cycloalkyl groups at the C-3α position. The inhibitor **XIX**, with the small cyclopropyl group, exhibited the highest activity, and the larger-sized cyclopentyl group in compound **XX** was also tolerated by the enzyme pocket. Compound **XXI** used the larger diisobutyl moiety at the C-6 position as compared to compounds **XVII**−**XX**. A comparison with compound **XVII** would suggest that the larger diisobutyl substitution at C-6 in compound **XXI** resulted in a noticeable reduction in inhibitory activity. As previously noted in Table 1, compound **IX** with the benzyl groups at C-6 showed very poor enzyme inhibitory activity.

The crystal structure of HIV-1 protease complexed with the inhibitor **XVII** (K_i = 22 nM) was determined (Figure 2, bottom left), and the four substituents around the 5,6-dihydro-4-hydroxy-2-pyrone ring, as expected, could be found to occupy the four enzyme pockets (the S_2−S_2' subsites). In this case, the complex crystallized in the space group $P6_122$ with only the protein monomer in the asymmetric unit. The inhibitor was located on the 2-fold axis, and it was difficult to determine the exact binding due to the overlap of the equally occupied sites. It appeared to be bound with the dihydropyrone ring between the two catalytic aspartic acid groups. Because of the overlap of the inhibitor on itself around the 2-fold axis, it is impossible to obtain a detailed model of the orientation of the inhibitor in the binding sight beyond determining that it fits into the four binding pockets, S2−S2′. The phenyl group does appear to extend into the expected S_2 subsite.

The structure−activity observation made from the compounds in Table 3 was that, unexpectedly, compounds with two identical and simple substituents at C-6 resulted in inhibitors with comparably good potency to compounds that contain the phenethyl group at C-6 in Table 2. Again, the crystallographic work with inhibitor **XVII** was not straightforward and resulted in additional subtle differences in inhibitor binding mode in the enzyme active site.

Table 4. HIV Protease Inhibitory Activity of Compounds with a Spirocycle at C-6



Continuing our structure-based design interest, we proposed to study compounds in which the two substituents at the C-6 position of the 5,6-dihydro-4-hydroxy-2-pyrone template formed a ring, leading to a conformationally constrained spiro ring system, which would also remove a chiral center at C-6 (see Table 4). Compounds **XXII**−**XXV** represented selected analogues in this series from a cyclopentyl to a cyclooctyl ring. The 6-membered ring in compound **XXIII** resulted in a reasonable inhibitor ($IC_{50} \approx 1$ μM), which was more active than compound **XXII**, with the 5-membered ring. Compounds **XXIV** and **XXV** with larger ring sizes were even less effective inhibitors.

Before the crystallographic structure of the HIV protease complexed with compound **XXIII** was determined, modeling for the cyclic analogues, based on crystallographic structural information of the acyclic analogues, suggested the possibility that a substituent on C-8 of the spiro ring system might be sufficiently close to form a hydrogen bond with the side group of Arg8A. A few analogues were then prepared in attempts to capitalize on this possibility. Compound **XXVI** contained a ketone functionality at this C-8 position, which was reduced to the corresponding hydroxyl group in compound **XXVII**, while the C-8 carbon atom was replaced with an oxygen atom in compound **XXVIII**. Contrary to expectation, all of these three compounds showed reduced inhibitory activity. The crystal structure of HIV-2 protease complexed with **XXIII** (K_i = 0.37 μM against HIV-2 protease) then provided a rationalization for why the compounds **XXVI**−**XXVIII** did not show anticipated increased binding affinity. Of the two enantiomers of **XXIII**, only the one with 3α*S* stereochemistry could be modeled into the difference electron density map. The template ring was bound nearly symmetrically to the catalytic aspartic acid residues (similar to **XVII**), with its ring puckering preferentially in the direction of the S_2' subsite (similar to **VI** and **VII**). This placed the cyclohexyl unit of the spirocyclic inhibitor extending into the S_2' subsite with its C-8 carbon far from the side chain of residue Arg8A. Even if the template ring were to pucker the other way (into the S_1' subsite), the compounds are not likely to provide for the H-bonding suggested by the modeling studies.

For analogue design based on structural chemistry information, another line of reasoning upon inspection of the crystal structure of compound **XXIII** in the enzyme active site led to the proposal that a benzyl substituent at the C-6 position of the spiro ring system might position the side chain in the S_1' subsite with improved binding affinity. Compound **XXIX** was then prepared but again, unfortunately, showed poorer inhibitory activity when compared to compound **XXIII**.

The structure−activity observation made from the compounds in Table 4 was that the bicyclic ring system resulted in compounds with poorer inhibitory activity. Attempts to improve binding affinity of these bicyclic compounds, based upon crystallographic information and molecular modeling work, by either the positioning of polar groups for hydrogen-bonding opportunity (compounds **XXVI**−**XXVIII**) or the addition of another substituent (compound **XXIX**) were not successful.

When tested, most of the compounds described in this report showed weak antiviral activity in cell-culture assays. Compound **XIX** (K_i = 15 nM), with a very simple chemical structure and the best enzyme inhibitory activity in these series of compounds, exhibited some antiviral activity in the HIV-1_{IIIB}-infected MT4 cells with an IC_{50} value of 5 μM. This level of activity was comparable to that observed for the 4-hydroxy-2-pyrone **II** (IC_{50} = 3 μM).[20] We continue to improve the enzyme inhibitory activity and, especially, the antiviral activity of this class of compounds, leading to compounds with potential therapeutic utility, results of which will be reported in due course.

Summary

From previous studies of the 4-hydroxy-2-pyrone class of nonpeptidic HIV protease inhibitors and from examination of the crystal structure of an early 4-hydroxy-2-pyrone inhibitor (compound **III**), we proposed that a corresponding 5,6-dihydro-4-hydroxy-2-pyrone template as represented by compound **IV** might allow the resulting two substituents at C-6 to readily extend into the S_1' and S_2' subsites and result in compounds with improved binding affinity. A series of 5,6-dihydro-4-hydroxy-2-pyrones were prepared with particular emphasis on the structure−activity relationship resulting from the variation at the two substituents at the C-6 position. The crystal structures of a number of representative inhibitors complexed with the HIV proteases were also determined to provide additional insight into the nature of the interaction between the enzyme and the modified inhibitors. It was interesting to note the unexpected positioning of the dihydropyrones in the enzyme active site as compared to the previously observed binding position of the pyrones. The flexibility of the dihydropyrone ring manifested itself in the puckering of the C-6 in two directions, resulting in different binding modes in the enzyme active site. This flexibility and puckering of the dihydropyrone ring likely influence the observation on the structure−activity relationship of analogues in these series. Although cocrystallization experiments with proteins were performed with diastereomeric mixtures, the structural data were made possible due to the specificity shown by the proteases to form crystals containing a single

stereoisomer out of a mixture of diastereomers. The six-point differences that span from S2′ through S2 subsites between the HIV-1 and HIV-2 proteases, in our opinion, are likely to only minimally influence the mode of binding of the different templates discovered in our laboratories. Many of the 5,6-dihydro-4-hydroxy-2-pyrone analogues prepared showed good enzyme inhibitory activity. In addition to having the required pharmacophore, the 4-hydroxy group with hydrogen-bonding interaction with the two catalytic aspartic acid residues and the lactone moiety replacing the ubiquitous water molecule in the active site, these inhibitors incorporated side chains that appropriately extended into the S_2-S_2' subsites of the enzyme active site. Compound **XIX**, a small nonpeptidic compound with a single chiral center, exhibited reasonable enzyme inhibitory activity (K_i = 15 nM) and showed antiviral activity (IC_{50} = 5 μM) in HIV-1_{IIIB}-infected MT4 cells. This result provided a direction of research for the discovery of much more active 5,6-dihydro-4-hydroxy-2-pyrones which will be reported in due course.

Experimental Section

Chemistry. Mass spectra, infrared spectra, and combustion analyses were obtained by the Structural, Analytical & Medicinal Chemistry Department of Pharmacia & Upjohn. ^{1}H NMR spectra were recorded at 300 MHz with a Bruker Model AM-300 spectrometer. Chemical shifts were reported as δ units relative to tetramethylsilane as internal standard. Thin-layer chromatography was conducted with Analtech 0.25-mm glass plates precoated with silica gel GF. Chromatography used E. Merck silica gel 60 (70−230 mesh for column chromatography and 230−400 mesh for flash chromatography). All solvents for chromatography were reagent grade.

Reagents were from commercial sources and used without further purification unless otherwise noted. Diethyl ether was Mallinkrodt anhydrous grade. Dichloromethane was dried over 4A molecular sieves. Tetrahydrofuran was distilled under argon from sodium metal in the presence of benzophenone.

5,6-Dihydro-4-hydroxy-6-phenethyl-6-propyl-2*H*-pyran-2-one (2). To a stirred suspension of 0.89 g (22.4 mmol) of sodium hydride (60% suspension in oil) in 20 mL of tetrahydrofuran at 0 °C was added 2.4 mL (22.4 mmol) of methyl acetoacetate. After 15 min, 14 mL (22.4 mmol) of *n*-butyllithium was added dropwise, and the reaction mixture was stirred for an additional 15 min. A solution of 2.0 g (11.4 mmol) of 1-phenyl-3-hexanone (**1**) in 5 mL of tetrahydrofuran was added to the solution of the reaction mixture over 5 min. The resulting reaction mixture was stirred for an additional 1 h, and then the reaction was quenched by the addition of saturated aqueous NH_4Cl. The mixture was extracted with several portions of ethyl acetate. The combined organic phase was washed with brine, dried ($MgSO_4$), and then evaporated under reduced pressure. The residue was dissolved in 100 mL of tetrahydrofuran, and 200 mL of 0.1 N aqueous NaOH was added. After stirring for 3 h, the mixture was washed with ethyl acetate, and the aqueous layer was adjusted to pH 3 with 1 N aqueous HCl and then extracted with several portions of dichloromethane. The combined organic phase was dried ($MgSO_4$) and then concentrated under reduced pressure to afford 1.96 g (67%) of 5,6-dihydro-4-hydroxy-6-phenethyl-6-propyl-2*H*-pyran-2-one (**2**) as a light yellow oil which solidified on standing: ^{1}H NMR ($CDCl_3$) δ 0.96 (t, 3H, J = 7.3 Hz), 1.48 (m, 2H), 1.72 (m, 2H), 1.98 (m, 2H), 2.73 (m, 4H), 3.43 (s, 2H), 7.15−7.32 (m, 5H). Anal. ($C_{16}H_{20}O_3$) C, H.

5,6-Dihydro-3(1,3-diphenyl-2-propenyl)-4-hydroxy-6-phenethyl-6-propyl-2*H*-pyran-2-one (XV). To a solution of 100 mg (0.38 mmol) of 5,6-dihydro-4-hydroxy-6-phenethyl-6-propyl-2*H*-pyran-2-one (**2**) and 161 mg (0.76 mmol) of 1,3-diphenyl-2-propen-1-ol in 2 mL of dioxane under argon was added 237 μL of BF_3-Et_2O. The resulting mixture was stirred at room temperature for 5 min, and then the reaction was quenched with water. The mixture was then extracted with several portions of ether, and the combined organic phase was extracted with several portions of 0.1 N aqueous NaOH. The combined aqueous phase was cooled to 0 °C and acidified to pH 1 by the dropwise addition of 2 N aqueous HCl. The milky solution was extracted with several portions of dichloromethane, and the combined organic phase was washed with saturated aqueous NaCl, dried (Na_2SO_4), and then concentrated under reduced pressure to give 155 mg (89%) of 5,6-dihydro-3-(1,3-diphenyl-2-propenyl)-4-hydroxy-6-phenethyl-6-propyl-2*H*-pyran-2-one (**XV**) as a white foam: ^{1}H NMR ($CDCl_3$) δ 0.90−1.02 (m, 3H), 1.38−1.53 (m, 2H), 1.73−2.18 (m, 4H), 2.49−2.75 (m, 4H), 5.23 (m, 1H), 6.36−6.75 (m, 2H), 7.04−7.41 (m, 15H); MS *m*/*z* 453, 331, 201, 175, 173, 115, 105, 91.

5,6-Dihydro-3-(1,3-diphenylpropyl)-4-hydroxy-6-phenethyl-6-propyl-2*H*-pyran-2-one (XVI). To a solution of 100 mg (0.22 mmol) of 5,6-dihydro-3-(1,3-diphenyl-2-propenyl)-4-hydroxy-6-phenethyl-6-propyl-2*H*-pyran-2-one (**XV**) in 5 mL of methanol was added 10 mg of 10% palladium on carbon. The resulting mixture was shaken under 40 psi of hydrogen gas for 10 h. The mixture was filtered through Celite and the solvent removed under reduced pressure. The residue was chromatographed on silica gel to yield 100 mg (100%) of 5,6-dihydro-3-(1,3-diphenylpropyl)-4-hydroxy-6-phenethyl-6-propyl-2*H*-pyran-2-one (**XVI**) as a white foam: ^{1}H NMR ($CDCl_3$) δ 0.72−1.03 (m, 3H), 1.17−2.78 (m, 14H), 4.36 (m, 1H), 5.84 (bs, 1H), 6.97−7.45 (m, 15H); HRMS calcd for $C_{31}H_{34}O_3$ + H_1 455.2586, found 455.2609.

Methyl 3-Oxo-2-(1-phenylpropyl)butanoate (4). To a stirred suspension of 0.36 g (9.0 mmol) of sodium hydride (60% suspension in oil) in 15 mL of tetrahydrofuran at 0 °C under argon was slowly added 0.86 mL (8.0 mmol) of methyl acetoacetate. After 15 min, the resulting cloudy tan solution was treated with 1.91 g (9.6 mmol) of α-ethylbenzyl bromide (**3**) (prepared from the corresponding commercially available alcohol and hydrogen bromide) as a solution in 5 mL of tetrahydrofuran. The mixture was warmed to 65 °C for 48 h and then cooled to room temperature. The reaction mixture was partitioned between diethyl ether and 1 N aqueous hydrochloric acid, and the aqueous phase was extracted with several portions of diethyl ether. The combined organic phase was washed with saturated aqueous NaCl, dried ($MgSO_4$), and then concentrated under reduced pressure. The resulting residue was purified by flash column chromatography on silica gel eluting with 40−50% diethyl ether in hexane to afford 0.53 g (2.3 mmol, 28%) of methyl 3-oxo-2-(1-phenylpropyl)butanoate (**4**) as a clear, colorless oil: ^{1}H NMR ($CDCl_3$) δ 0.70 and 0.90 (t, 3H), 1.5−1.85 (m, 2H), 1.90 and 2.30 (s, 3H), 3.28 (m, 1H), 3.38 and 3.76 (s, 3H), 3.8−3.9 (m, 1H), 7.1−7.3 (m, 5H).

Methyl 5-Hydroxy-3-oxo-5-phenyl-2-(1-phenylpropyl)-heptanoate (5). To a stirred suspension of 0.10 g (2.5 mmol) of sodium hydride (60% suspension in oil) in 5 mL of tetrahydrofuran at 0 °C under argon was slowly added 0.53 g (2.3 mmol) of 3-oxo-2-(1-phenylpropyl)butanoate (**4**) in 5 mL of tetrahydrofuran. After 15 min, the tan suspension was warmed to room temperature. After 1 h, the resulting cloudy solution was recooled to 0 °C and treated dropwise with 1.55 mL (2.5 mmol) of *n*-butyllithium (1.6 M in hexane). After 15 min, the reaction mixture was treated with 0.33 mL (2.5 mmol) of propiophenone. The mixture was stirred for an additional 30 min; then the reaction was quenched with 1 mL of concentrated hydrochloric acid. The mixture was partitioned between diethyl ether and water, and the aqueous phase was extracted with several portions of diethyl ether. The combined organic phase was washed with saturated aqueous NaCl, dried ($MgSO_4$), and then concentrated under reduced pressure. The resulting residue was purified by flash column chromatography on silica gel eluting with 5−15% ethyl acetate in hexane to afford 0.48 g (1.3 mmol, 58%) of methyl 5-hydroxy-3-oxo-5-phenyl-2-(1-phenylpropyl)heptanoate (**5**) as a pale, tan oil: ^{1}H NMR ($CDCl_3$) δ 0.5−1.0 (m, 6H), 1.2−1.9 (m, 4H), 2.9−4.6 (m, 4H), 3.24 and 3.32 (s, 3H), 7.0−7.4 (m, 10H); EI-MS *m*/*z* 339, 221, 189, 135, 119, 105, 91.

5,6-Dihydro-6-ethyl-4-hydroxy-6-phenyl-3-(1-phenylpropyl)-2*H*-pyran-2-one (V). To a stirring solution of 0.15 g (0.40 mmol) of methyl 5-hydroxy-3-oxo-5-phenyl-2-(1-phenylpropyl)heptanoate (**5**) in 5 mL of tetrahydrofuran was added 40 mL of 0.1 N aqueous sodium hydroxide. After 4 h,

the solution was concentrated under reduced pressure to remove most of the tetrahydrofuran. The aqueous phase was washed with diethyl ether and then cooled to 0 °C and acidified with 2 N aqueous hydrochloric acid. The resulting cloudy suspension was extracted with several portions of diethyl ether; these extracts were washed with saturated aqueous NaCl, dried ($MgSO_4$), and then concentrated under reduced pressure to afford 0.069 g (0.21 mmol, 51%) of 5,6-dihydro-6-ethyl-4-hydroxy-6-phenyl-3-(1-phenylpropyl)-2*H*-pyran-2-one (**V**): ^{1}H NMR ($CDCl_3$) δ 0.7−0.8 (m, 6H), 1.5−2.0 (m, 4H), 3.0 (m, 2H), 3.9 (m, 1H), 7.1−7.3 (m, 10H); HRMS calcd for $C_{22}H_{24}O_3$ 336.1725, found 336.1722.

Methyl 1-Phenyl-3-(trimethylsilyl)-(2*E*)-propenyl Carbonate (7). To a stirred solution of 116.4 mL (198 mmol) of *tert*-butyllithium (1.7 M in pentane) in 188 mL of tetrahydrofuran at −78 °C under nitrogen was added 15.18 mL (99 mmol) of commercially available (2-bromovinyl)trimethylsilane (**6**) over 10 min. After an additional 15 min, 10 g (94 mmol) of benzaldehyde was added. After another 45 min, 7.64 mL (99 mmol) of freshly distilled methyl chloroformate was added. The resulting mixture was allowed to warm to room temperature and stirred for 2 h. It was then poured into water and extracted with several portions of ethyl acetate. The combined organic phase was washed with saturated aqueous NaCl, dried over Na_2SO_4, and then concentrated under reduced pressure. The resulting residue was chromatographed on a silica gel column eluting with 1−2% ethyl acetate in hexane to give 21.5 g (81 mmol, 86%) of methyl 1-phenyl-3-(trimethylsilyl)-(2*E*)-propenyl carbonate (**7**) as an oil: ^{1}H NMR ($CDCl_3$) δ 0.03 (s, 9H), 3.71 (s, 3H), 5.90 (dd, 1H, J = 1.3, 18.6 Hz), 5.99 (dd, 1H, J = 1.3, 5.1 Hz), 6.10 (dd, 1H, J = 5.1, 18.6 Hz), 7.18−7.33 (m, 5H); MS *m*/*z* 264, 235, 205, 145, 131, 116, 115; HRMS calcd for $C_{14}H_{20}O_3Si$ 264.1182, found 264.1189.

5,6-Dihydro-4-hydroxy-6-phenyl-6-propyl-2*H*-pyran-2-one (8). To a stirred suspension of 3.03 g (75.8 mmol) of sodium hydride (60% dispersion in mineral oil) in 60 mL of tetrahydrofuran at 0 °C was added 8.0 g (68.9 mmol) of methyl acetoacetate. After 15 min, 47.4 mL (75.8 mmol) of *n*-butyllithium (1.6 M in hexane) was added dropwise. After an additional 15 min, 11.5 mL (79.2 mmol) of butyrophenone in 5 mL of tetrahydrofuran was added. After 1 h, the reaction mixture was poured into saturated aqueous $(NH_4)_2SO_4$ and then extracted with several portions of dichloromethane. The combined organic phase was dried over Na_2SO_4 and then concentrated under reduced pressure. The resulting residue was dissolved in 180 mL of tetrahydrofuran, and 680 mL of 0.1 N NaOH was added. After stirring for 2 h, the mixture was washed with ethyl acetate. The aqueous phase was then adjusted to pH 3 with hydrochloric acid and then extracted with several portions of chloroform/methanol. The combined organic phase was dried over $MgSO_4$ and then concentrated under reduced pressure to give 12.54 g (54 mmol, 78%) of 5,6-dihydro-4-hydroxy-6-phenyl-6-propyl-2*H*-pyran-2-one (**8**) as a white solid: ^{1}H NMR ($CDCl_3$) δ 0.87 (t, 3H, J = 7.3 Hz), 1.25−1.33 (m, 2H), 1.95 (t, 2H, J = 8.4 Hz), 2.88 (d, 1H, J = 17.4 Hz), 2.91 (d, 1H, J = 20.4 Hz), 3.23 (d, 1H, J = 20.4 Hz), 3.35 (d, 1H, J = 17.4 Hz), 7.25−7.40 (m, 5H); IR (mineral oil mull) 1663, 1635, 1592, 1582, 1450, 1342, 1332, 1319, 1285, 1263, 1244 cm^{-1}; MS *m*/*z* 232, 189, 149, 147, 105.

5,6-Dihydro-4-hydroxy-6-phenyl-3-[1-phenyl-3-(trimethylsilyl)-(2*E*)-propenyl]-6-propyl-2*H*-pyran-2-one (9). To a stirred solution of 2.5 g (10.8 mmol) of 5,6-dihydro-4-hydroxy-6-phenyl-6-propyl-2*H*-pyran-2-one (**8**) and 4.4 g (11.8 mmol) of methyl 1-phenyl-3-(trimethylsilyl)-(2*E*)-propenyl carbonate (**7**) in 50 mL of distilled toluene under nitrogen were added 120 mg (0.5 mmol) of palladium acetate and 565 mg (2 mmol) of triphenylphosphine. The resulting mixture was heated at 70 °C for 1.5 h. The cooled reaction mixture was poured into water and then extracted with several portions of ethyl acetate. The combined organic phase was dried over Na_2SO_4 and then concentrated under reduced pressure. The residue was chromatographed on a silica gel column eluting with 25% ethyl acetate in hexane to give 2.68 g (6.4 mmol, 60%) of 5,6-dihydro-4-hydroxy-6-phenyl-3-[1-phenyl-3-(trimethylsilyl)-(2*E*)-propenyl]-6-propyl-2*H*-pyran-2-one (**9**): ^{1}H NMR ($CDCl_3$) δ 0.0 and 0.10 (s, 9H, 4:6 ratio of diastereomers), 0.89 and 0.91 (t, 3H, J = 7.3 Hz), 1.20−1.52 (m, 2H), 1.90−2.05 (m, 2H), 2.93−3.18 (m, 2H), 5.03−5.10 (m, 1H), 5.62−6.74 (m, 2H), 7.05−7.44 (m, 10H); MS *m*/*z* 420, 402, 377, 303, 287, 274, 230, 184, 173, 73.

5,6-Dihydro-4-hydroxy-6-phenyl-3-(1-phenyl-2-propenyl)-6-propyl-2*H*-pyran-2-one (10). To a stirred solution of 2.65 g (6.3 mmol) of 5,6-dihydro-4-hydroxy-6-phenyl-3-[1-phenyl-3-(trimethylsilyl)-(2*E*)-propenyl]-6-propyl-2*H*-pyran-2-one (**9**) in 12 mL of acetonitrile was added 600 mg (3.16 mmol) of *p*-toluenesulfonic acid. The resulting mixture was heated to reflux for 1.5 h. The cooled reaction mixture was poured into water and then extracted with several portions of ethyl acetate. The combined organic phase was washed with saturated NaCl, dried over Na_2SO_4, and then concentrated under reduced pressure to give 2.21 g (6.3 mmol, 100%) of 5,6-dihydro-4-hydroxy-6-phenyl-3-(1-phenyl-2-propenyl)-6-propyl-2*H*-pyran-2-one (**10**) which was carried on to the next reaction without further purification: MS *m*/*z* 348, 330, 305, 287, 277, 230, 184, 173, 146, 131, 117.

5,6-Dihydro-4-hydroxy-6-phenyl-3-(1-phenylpropyl)-6-propyl-2*H*-pyran-2-one (VI). To 2.2 g (6.3 mmol) of 5,6-dihydro-4-hydroxy-6-phenyl-3-(1-phenyl-2-propenyl)-6-propyl-2*H*-pyran-2-one (**10**) in 20 mL of ethanol was added 0.4 g of 10% palladium hydroxide on carbon. The resulting mixture was shaken under 40 psi of hydrogen gas for 8 h and then filtered through Celite. The residue was crystallized from acetonitrile to give 0.95 g of one diastereomer, whereas the balance of the material remained a mixture of the two diastereomers. ^{1}H NMR indicated one diastereomer for the crystallized material (43%): mp 197−198 °C; ^{1}H NMR (DMSO-d_6) δ 0.50 (t, 3H, J = 7 Hz), 0.77 (t, 3H, J = 7 Hz), 0.90−1.08 (m, 1H), 1.10−1.30 (m, 1H), 1.65−2.05 (m, 4H), 3.07 (q, 2H, J = 17, 25 Hz), 3.68−3.72 (m, 1H), 6.98−7.12 (m, 5H), 7.23−7.35 (m, 5H); IR (mineral oil mull) 1642, 1603, 1595, 1575, 1448, 1329, 1317, 1276 cm^{-1}; MS *m*/*z* 350, 332, 306, 277, 173, 159, 164. Anal. ($C_{22}H_{26}O_3$) C, H.

5,6-Dihydro-6-cyclohexyl-4-hydroxy-3-[1-(3-hydroxyphenyl)propyl]-6-phenyl-2*H*-pyran-2-one (VII): ^{1}H NMR (DMSO-d_6) δ 0.20 and 0.21 (t, 3H, J = 7.3 Hz, 7:3 ratio of diastereomers), 0.69−1.18 (m, 4H), 1.50−1.95 (m, 8H), 2.98−3.15 (m, 2H), 3.45−3.83 (m, 1H), 6.32−6.90 (m, 4H), 7.20−7.32 (m, 5H), 8.99−9.01 (s, 1H, 3:7 ratio of diastereomers); IR (mineral oil mull) 1645, 1616, 1599, 1588, 1448, 1334, 1260, 1255, 1235, 1160 cm^{-1}; MS *m*/*z* 406, 388, 362, 323, 203, 186, 172, 147, 135, 105; HRMS calcd for $C_{26}H_{30}O_4$ 406.2153, found 406.2144. Anal. ($C_{26}H_{30}O_4 \cdot 0.33C_4H_8O_2$) C, H.

5,6-Dihydro-4-hydroxy-6-phenyl-6-(phenylmethyl)-3-(1-phenylpropyl)-2*H*-pyran-2-one (VIII): ^{1}H NMR ($CDCl_3$) δ 0.42 and 0.94 (m, 3H), 1.30−2.10 (m, 2H), 2.77−3.34 (m, 4H), 3.91 and 4.13 (m, 1H), 6.86−7.49 (m, 15H); HRMS calcd for $C_{27}H_{26}O_3 + H_1$ 399.1960, found 399.1598.

5,6-Dihydro-6,6-bis(phenylmethyl)-4-hydroxy-3-(1-phenylpropyl)-2*H*-pyran-2-one (IX): ^{1}H NMR ($CDCl_3$−CD_3OD) δ 0.95 (t, 3H, J = 7.3 Hz), 2.1 (m, 2H), 2.42 (s, 2H), 2.8−3.1 (m, 4H), 4.11 (t, 1H), 7.0−7.5 (m, 15H): HRMS calcd for $C_{28}H_{28}O_3$ 412.2038, found 412.2032.

6-Butyl-5,6-dihydro-4-hydroxy-6-(phenylmethyl)-3-(1-phenylpropyl)-2*H*-pyran-2-one (X): ^{1}H NMR ($CDCl_3$−CD_3OD) δ 0.80−0.95 (m, 6H), 1.2−1.7 (m, 6H), 2.0−2.3 (m, 2H), 2.45 (s, 2H), 2.96 (s, 2H), 4.06 (t, 1H, J = 8.0 Hz), 7.0−7.5 (m, 10H); HRMS calcd for $C_{25}H_{30}O_3$ 378.2195, found 378.2205.

5,6-Dihydro-4-hydroxy-6-phenethyl-3-(1-phenyl-2-propenyl)-6-propyl-2*H*-pyran-2-one (XI): ^{1}H NMR ($CDCl_3$) δ 0.87−1.04 (m, 3H), 1.34−1.55 (m, 2H), 1.68−1.77 (m, 2H), 1.77−2.14 (m, 2H), 2.45−2.77 (m, 4H), 5.06 (m, 2H), 5.40−5.47 (m, 1H), 6.30−6.45 (m, 1H), 6.56−6.62 (m, 1H), 7.08−7.38 (m, 10H); HRMS calcd for $C_{25}H_{28}O_3 + H_1$ 377.2117, found 377.2128.

5,6-Dihydro-4-hydroxy-6-phenethyl-3-(1-phenylpropyl)-6-propyl-2*H*-pyran-2-one (XII): ^{1}H NMR ($CDCl_3$) δ 0.87−1.13 (m, 6H), 1.25−2.20 (m, 8H), 2.33−2.54 (m, 2H), 2.60−2.78 (m, 4H), 4.23 (m, 1H), 5.68 (bs, 1H), 7.13−7.42 (m, 10H); HRMS calcd for $C_{25}H_{30}O_3 + H_1$ 379.2273, found 379.2264.

4-Hydroxy-1,10,13-trioxadispiro[5.2.4.2]pentadec-3-en-2-one (11). To a stirred suspension of 1.82 g (38 mmol) of sodium hydride (50% dispersion in mineral oil) in 60 mL of tetrahydrofuran at 0 °C was added 3.7 mL (34 mmol) of methyl acetoacetate. After 15 min, 24 mL (38 mmol) of *n*-butyllithium

(1.6 M in hexane) was added dropwise. After an additional 15 min, 6.56 g (42 mmol) of 1,4-cyclohexanedione monoethylene ketal in 5 mL of tetrahydrofuran was added dropwise, and the mixture stirred at room temperature for 1 h. The reaction mixture was poured into saturated aqueous NH_4Cl and then extracted with several portions of dichloromethane. The combined organic phase was dried over Na_2SO_4 and then concentrated under reduced pressure. The resulting residue was dissolved in 100 mL of tetrahydrofuran, and 350 mL of 0.1 N NaOH was added. After stirring for 2 h, the mixture was acidified with concentrated hydrochloric acid and extracted with several portions of dichloromethane. The combined organic phase was dried over Na_2SO_4 and then concentrated under reduced pressure. The resulting residue was chromatographed on a silica gel column eluting with 5% methanol in dichloromethane to give 1.8 g (7.5 mmol, 22%) of 4-hydroxy-1,10,13-trioxadispiro[5.2.4.2]pentadec-3-en-2-one (**11**) as a white solid after crystallization from toluene/petroleum ether: ^{1}H NMR ($CDCl_3$) δ 1.6−2.1 (m, 8H), 2.68 (s, 2H), 3.44 (s, 2H), 3.9−4.0 (m, 4H); IR (mull) 1638, 1581, 1488, 1407, 1344, 1330, 1324, 1288, 1258, 1242, 1220, 1095, 1038, 1018, 999 cm^{-1}; MS (EI) *m/z* 240 (M^+) 100, 99, 86, 84, 56, 55, 42, 41, 40, 38. Anal. ($C_{12}H_{16}O_5$) C, H.

4-Hydroxy-3-[1-phenyl-3-(trimethylsilyl)-2-propenyl]-1,10,13-trioxadispiro[5.2.4.2]pentadec-3-en-2-one (12). To a stirred solution of 150 mg (0.62 mmol) of 4-hydroxy-1,10,-13-trioxadispiro[5.2.4.2]pentadec-3-en-2-one (**11**) and 180 mg (0.68 mmol) of methyl 1-phenyl-3-(trimethylsilyl)-(2*E*)-propenyl carbonate (**7**) in 5 mL of toluene under nitrogen were added 10 mg (0.04 mmol) of palladium acetate and 33 mg (0.12 mmol) of triphenylphosphine. The resulting mixture was heated at 70 °C for 2 h. The cooled reaction mixture was poured into water and then extracted with several portions of ethyl acetate. The combined organic phase was dried over Na_2SO_4 and then concentrated under reduced pressure. The residue was chromatographed on a silica gel column eluting with 20% ethyl acetate in dichloromethane to give 225 mg (0.52 mmol, 84%) of 4-hydroxy-3-[1-phenyl-3-(trimethylsilyl)-2-propenyl]-1,10,-13-trioxadispiro[5.2.4.2]pentadec-3-en-2-one (**12**): ^{1}H NMR ($CDCl_3$) δ 0.08 (s, 9H), 1.4−2.2 (m, 10H), 3.8 (m, 4H), 5.0 (m, 1H), 5.6 (dd, J = 2.3, 19.0 Hz, 1H), 6.4 (dd, J = 5.3, 15.7 Hz, 1H), 6.55 (s, 1H), 7.1−7.4 (m, 5H); MS (EI) *m/z* 428 (M^+), 157, 154, 153, 140, 99, 86, 75, 73, 55, 43; HRMS (EI) calcd for $C_{24}H_{32}O_5Si$ 428.2019, found 428.2017.

4-Hydroxy-3-(1-phenyl-2-propenyl)-1,10,13-trioxadispiro[5.2.4.2]pentadec-3-en-2-one (13). To a stirred solution of 210 mg (0.5 mmol) of 4-hydroxy-3-[1-phenyl-3-(trimethylsilyl)-2-propenyl]-1,10,13-trioxadispiro[5.2.4.2]-pentadec-3-en-2-one (**12**) in 5 mL of acetonitrile was added 50 mg (0.26 mmol) of *p*-toluenesulfonic acid. The resulting mixture was heated at reflux for 2 h. The cooled reaction mixture was poured into water and then extracted with several portions of ethyl acetate. The combined organic phase was washed with saturated NaCl, dried over Na_2SO_4, and then concentrated under reduced pressure. The residue was chromatographed on a silica gel column eluting with 20% ethyl acetate in dichloromethane to give 90 mg (0.25 mmol, 52%) of 4-hydroxy-3-(1-phenyl-2-propenyl)-1,10,13-trioxadispiro[5.2.4.2]-pentadec-3-en-2-one (**13**): ^{1}H NMR ($CDCl_3$) δ 1.2−2.2 (m, 10H), 2.52 (d, J = 5.0 Hz, 2H), 3.8−4.0 (m, 4H), 5.0−5.4 (m, 3H), 6.3 (m, 1H), 6.73 (s, 1H), 7.1−7.4 (m, 5H); MS (EI) *m/z* 356 (M^+), 158, 154, 153, 140, 117, 115, 99, 86, 55; HRMS (EI) calcd for $C_{21}H_{24}O_5$ 356.1624, found 356.1614.

4-Hydroxy-3-(1-phenyl-2-propyl)-1,10,13-trioxadispiro-[5.2.4.2]pentadec-3-en-2-one (14). To 85 mg (0.24 mmol) of 4-hydroxy-3-(1-phenyl-2-propenyl)-1,10,13-trioxadispiro[5.2.4.2]-pentadec-3-en-2-one (**13**) in 10 mL of ethanol was added 30 mg of 10% palladium hydroxide on carbon. The resulting mixture was shaken under 40 psi of hydrogen gas for 4 h and then filtered through Celite and the filtrate concentrated under reduced pressure to give 80 mg (0.22 mmol, 94%) of 4-hydroxy-3-(1-phenyl-2-propyl)-1,10,13-trioxadispiro[5.2.4.2]pentadec-3-en-2-one (**14**): ^{1}H NMR ($CDCl_3$) δ 0.8−1.0 (m, 3H), 1.1−2.5 (m, 11H), 3.0−3.7 (m, 3H), 3.8−4.0 (m, 4H), 7.1−7.4 (m, 5H); MS (EI) *m/z* 358 (M^+), 256, 140, 119, 118, 101, 99, 91, 86, 55, 40; HRMS (EI) calcd for $C_{21}H_{26}O_5$ 358.1780, found 358.1786.

4-Hydroxy-8-oxo-3-(1-phenylpropyl)-1-oxaspiro[5.5]-undec-3-en-2-one (XXVI). A solution of 80 mg (0.22 mmol) of 4-hydroxy-3-(1-phenyl-2-propyl)-1,10,13-trioxadispiro[5.2.4.2]-pentadec-3-en-2-one (**14**) in 2 mL of tetrahydrofuran was treated with 1 mL of 1 N hydrochloric acid and then stirred at room temperature for 18 h. The solution was diluted with ethyl acetate, washed with water and saturated NaCl, and dried over Na_2SO_4, and the resulting residue was chromatographed on a silica gel column eluting with 40% ethyl acetate in hexane with 1% acetic acid to give 40 mg (0.13 mmol, 57%) of 4-hydroxy-8-oxo-3-(1-phenylpropyl)-1-oxaspiro[5.5]undec-3-en-2-one (**XXVI**): ^{1}H NMR (CD_3OD) δ 0.8 (m, 3H), 1.5−2.7 (m, 13H), 3.9 (m, 1H), 6.9−7.3 (m, 5H); MS (EI) *m/z* 314 (M^+), 176, 159, 158, 131, 119, 118, 103, 91, 77, 40; HRMS (EI) calcd for $C_{19}H_{22}O_4$ 314.1518, found 314.1524.

4,8-Dihydroxy-3-(1-phenylpropyl)-1-oxaspiro[5.5]undec-3-en-2-one (XXVII). A solution of 100 mg (0.32 mmol) of 4-hydroxy-3-oxo-3-(1-phenylpropyl)-1-oxaspiro[5.5]undec-3-en-2-one (**XXVI**) in 3 mL of methanol at 5 °C was treated with 15 mg (0.4 mmol) of sodium borohydride and then stirred at room temperature for 2 h. An additional 15 mg of sodium borohydride was added, and stirring continued for 2 h. The resulting mixture was diluted with water and acidified with 1 N hydrochloric acid followed by extraction with several portions of ethyl acetate. The combined organic phase was washed with saturated NaCl and dried over Na_2SO_4, and the resulting residue was chromatographed on a silica gel column eluting with 50% ethyl acetate in dichloromethane to give 100 mg (0.31 mmol, 99%) of 4,8-dihydroxy-3-(1-phenylpropyl)-1-oxaspiro[5.5]undec-3-en-2-one (**XXVII**): ^{1}H NMR (CD_3OD) δ 0.9 (t, 3H, J = 8.0 Hz), 1.4−2.3 (m, 13H), 2.52 (s, 1H), 3.5 (m, 1H), 4.0 (m, 1H), 7.0−7.4 (m, 5H); MS (EI) *m/z* 316 (M^+), 177, 159, 158, 131, 119, 118, 103, 94, 91, 79; HRMS (EI) calcd for $C_{19}H_{24}O_4$ 316.1674, found 316.1676.

4-Hydroxy-3-(1-phenyl-2-propenyl)-1-oxaspiro[5.4]dec-3-en-2-one (XXII): ^{1}H NMR ($CDCl_3$) δ 1.57 (m, 4H), 1.76 (m, 2H), 1.96 (m, 2H), 2.47 (d, 1H, J = 17.1 Hz), 2.57 (d, 1H, J = 17.1 Hz), 4.87 (d, 1H, J = 6.4 Hz), 4.99 (dt, 1H, J = 1.7, 17.3 Hz), 5.22 (dt, 1H, J = 1.7, 10.1 Hz), 6.31 (ddd, 1H, J = 6.4, 10.2, 17.3 Hz), 7.20 (m, 5H), 7.76 (bs, 1H); IR (mull) 3024, 2612, 1660, 1601, 1495, 1351, 1350, 1336, 1307, 1284, 1260, 1139, 1113, 925, 724 cm^{-1}; MS (EI) *m/z* 284, 175, 158, 157, 129, 128, 117, 115, 91, 77, 55; HRMS (EI) calcd for $C_{18}H_{20}O_3$ 284.1412, found 284.1411. Anal. ($C_{18}H_{20}O_{.}0.1H_2O$) C, H.

4-Hydroxy-3-(1-phenylpropyl)-1-oxaspiro[5.5]undec-3-en-2-one, sodium salt (XXIII): ^{1}H NMR (CD_3OD) δ 1.08 (t, 3H, J = 7.3 Hz), 1.50−1.78 (m, 6H), 1.83−1.96 (m, 2H), 2.02−2.23 (m, 1H), 2.35−2.46 (m, 1H), 2.52 (s, 2H), 4.23 (m, 1H), 7.16 (t, 1H, J = 7.3 Hz), 7.30 (t, 2H, J = 7.4 Hz), 7.55 (d, 2H, J = 7.6 Hz); IR (mineral oil mull) 1629, 1515, 1450, 1421, 1409, 1365, 1341, 1310 cm^{-1}; MS *m/z* 323. Anal. ($C_{19}H_{23}O_3Na{\cdot}0.5H_2O$) C, H.

4-Hydroxy-3-(1-phenyl-2-propenyl)-1-oxaspiro[5.6]-dodec-3-en-2-one (XXIV): ^{1}H NMR (CD_3OD) δ 1.50−2.15 (m, 12H), 2.74 (s, 2H), 4.98 (d, 1H, J = 8.3 Hz), 5.26 (m, 1H), 6.64 (ddd, 1H, J = 8.3, 10.1, 16.9 Hz), 7.23−7.45 (m, 5H); MS (EI) *m/z* 312, 198, 175, 158, 157, 137, 129, 117, 115, 91, 55; HRMS (EI) calcd for $C_{20}H_{24}O_3$ 312.1725, found 312.1724. Anal. ($C_{20}H_{24}O_3$) C, H.

4-Hydroxy-3-(1-phenylpropyl)-1-oxaspiro[5.7]tridec-3-en-2-one (XXV): ^{1}H NMR ($CDCl_3$) δ 0.95 (t, 3H, J = 8.3 Hz), 1.3−1.7 (m, 13H), 2.0−2.2 (m, 4H), 2.48 (s, 2H), 4.12 (t, 1H, J = 8.7 Hz), 7.1−7.4 (m, 5H); MS (EI) *m/z* 328 (M^+), 177, 176, 159, 131, 119, 118, 96, 91, 55, 40; HRMS (EI) calcd for $C_{21}H_{28}O_3$ 328.2038, found 328.2039.

4-Hydroxy-3-(1-phenyl-2-propenyl)-1,8-dioxaspiro[5.5]-undec-3-en-2-one (XXVIII): ^{1}H NMR ($CDCl_3$) δ 1.5−2.1 (m, 5H), 2.55 (d, 2H, J = 8.1 Hz), 3.7−4.0 (m, 4H), 5.0−5.5 (m, 3H), 6.3−6.4 (m, 1H), 7.2−7.5 (m, 5H); MS (EI) *m/z* 300 (M^+), 282, 175, 158, 157, 129, 128, 117, 115, 91, 83.

4-Hydroxy-6-(phenylmethyl)-3-(1-phenyl-2-propenyl)-1-oxaspiro[5.5]undec-3-en-2-one (XXIX): ^{1}H NMR ($CDCl_3$) δ 1.25−1.72 (m, 9H), 2.11−2.43 (m, 2H), 2.90−3.11 (m, 2H), 4.99 (m, 2H), 5.28 (dt, 1H, J = 0.9, 10.3 Hz), 6.27 (m, 1H), 6.90−7.35 (m, 10H); MS (EI) *m/z* 388, 297, 279, 172, 171, 131, 129, 117, 115, 91, 84; HRMS (EI) calcd for $C_{26}H_{28}O_3$ 388.2038, found 388.2033.

5,6-Dihydro-6,6-diisobutyl-3-(1-phenylpropyl)-4-hydroxy-2*H*-pyran-2-one (XXI). To a solution of 150 mg (0.66 mmol) of 5,6-dihydro-6,6-diisobutyl-4-hydroxy-2*H*-pyran-2-one (**15**) and 69 μL (0.68 mmol) of benzaldehyde in 3 mL of dry tetrahydrofuran at 0 °C was added 150 mg of $AlCl_3$. The cooling bath was removed, and the yellow solution was allowed to stir at room temperature for 2 h. The reaction was quenched by the addition of solid $Na_2CO_3 \cdot 10H_2O$ and the mixture vigorously stirred for 5 min. The mixture was filtered through Celite with ether and the filtrate evaporated to dryness under reduced pressure. To the resulting benzylidene intermediate **16** in 3 mL of dry tetrahydrofuran was added 40 mg of $CuBr-Me_2S$, and a solution of 0.73 mL of Et_3Al (1 M in hexane) was added at room temperature dropwise. The color changed from yellow to dark green when the reaction was completed. The reaction was quenched by the addition of water, and the reaction mixture was extracted with several portions of ether. The combined organic phase was washed with brine, dried ($MgSO_4$), and concentrated under reduced pressure. The residue was chromatographed on a silica gel column with 25% ethyl acetate in hexane to give 78 mg (34%) of 5,6-dihydro-6,6-diisobutyl-3-(1-phenylpropyl)-4-hydroxy-2*H*-pyran-2-one (**XXI**) as a white solid: ^{1}H NMR ($CDCl_3$) δ 0.85−1.01 (m, 15H), 1.48−1.54 (m, 2H), 1.59−1.68 (m, 4H), 1.98−2.12 (m, 1H), 2.12−2.27 (m, 1H), 2.49 (s, 2H), 4.03 (m, 1H), 7.13−7.31 (t, 3H, J = 7.7 Hz), 7.40 (d, 2H, J = 7.2 Hz); HRMS calcd for $C_{22}H_{32}O_3 + H_1$ 345.2430, found 345.2454. Anal. ($C_{22}H_{32}O_3$) C, H.

5,6-Dihydro-3-(α-cyclopropylphenylmethyl)-4-hydroxy-6-phenethyl-6-propyl-2*H*-pyran-2-one (XIII). To a solution of 100 mg (0.38 mmol) of 5,6-dihydro-4-hydroxy-6-phenethyl-6-propyl-2*H*-pyran-2-one (**2**) and 39 μL (0.38 mmol) of benzaldehyde in 2 mL of dry tetrahydrofuran at 0 °C was added 90 mg of $AlCl_3$. The cooling bath was removed, and the yellow solution was allowed to stir at room temperature for 2 h. The reaction was quenched by the addition of solid $Na_2CO_3 \cdot 10H_2O$ and the mixture vigorously stirred for 5 min. The mixture was filtered through Celite with ether and the filtrate evaporated to dryness under reduced pressure. The resulting benzylidene intermediate **17** and 25 mg of $CuBr-Me_2S$ were dissolved in 2 mL of dry tetrahydrofuran and cooled to −78 °C. To the stirred reaction mixture was added a solution of 1.7 mL of cyclopropylmagnesium bromide (0.25 M in tetrahydrofuran) dropwise, and the resulting reaction mixture was stirred for 30 min. The reaction was quenched by the addition of water and neutralized by the addition of 0.1 N aqueous HCl. The reaction mixture was extracted with several portions of ether. The combined organic phase was washed with brine, dried ($MgSO_4$), and concentrated under reduced pressure. The resulting residue was chromatographed on a silica gel column with 25% ethyl acetate in hexane to provide 84 mg (63%) of 5,6-dihydro-3-(α-cyclopropylphenylmethyl)-4-hydroxy-6-phenethyl-6-propyl-2*H*-pyran-2-one (**XIII**) as a white foam: ^{1}H NMR ($CDCl_3$) δ 0.16 (m, 2H), 0.48 (m, 1H), 0.68 (m, 1H), 0.96 (m, 3H), 1.41 (m, 2H), 1.53−2.14 (m, 5H), 2.58−2.72 (m, 4H), 3.24 (m, 1H), 7.08−7.27 (m, 8H), 7.39 (m, 2H); HRMS calcd for $C_{26}H_{30}O_3 + H_1$ 391.2273, found 391.2287.

3-(α-Cyclopentylphenylmethyl)-5,6-dihydro-4-hydroxy-6-phenethyl-6-propyl-2*H*-pyran-2-one (XIV): ^{1}H NMR ($CDCl_3$) δ 0.87 (m, 3H), 1.04 (m, 1H), 1.21 (m, 3H), 1.47−1.92 (m, 9H), 1.94 (m, 1H), 2.41−2.62 (m, 4H), 3.15 (m, 1H), 3.80−3.86 (m, 2H), 7.09−7.25 (m, 8H), 7.48 (m, 2H); HRMS calcd for $C_{28}H_{34}O_3 + H_1$ 419.2586, found 419.2589. Anal. ($C_{28}H_{34}O_3$) C, H.

5,6-Dihydro-6,6-dipropyl-4-hydroxy-3-(1-phenylpropyl)-2*H*-pyran-2-one (XVII): ^{1}H NMR ($CDCl_3$) δ 0.90 (m, 9H), 1.30 (m, 4H), 1.58−1.69 (m, 4H), 2.08 (m, 2H), 2.41 (d, 1H, J = 17.3 Hz), 3.51 (d, 1H, J = 17.3 Hz), 4.15 (t, 1H, J = 7.8 Hz), 7.10−7.49 (m, 5H); HRMS calcd for $C_{20}H_{28}O_3$ 316.2038, found 316.2040.

3-(α-*tert*-Butylphenylmethyl)-5,6-dihydro-6,6-dipropyl-4-hydroxy-2*H*-pyran-2-one (XVIII): ^{1}H NMR ($CDCl_3$) δ 0.88 (t, J = 7.2 Hz, 6H), 1.07 (s, 9H), 1.3 (m, 4H), 1.5−1.7 (m, 4H), 2.48 (m, 1H), 4.02 (br, 2H), 4.17 (s, 1H), 7.1−7.2 (m, 3H), 7.55 (d, J = 7.8 Hz, 2H); FAB HRMS (M + H) calcd for $C_{22}H_{33}O_3$ 345.2430, found 345.2438.

3-(α-Cyclopropylphenylmethyl)-5,6-dihydro-6,6-dipropyl-4-hydroxy-2*H*-pyran-2-one (XIX): ^{1}H NMR ($CDCl_3$) δ 0.17 (m, 2H), 0.47 (m, 1H), 0.67 (m, 1H), 0.91 (m, 6H), 1.36 (m, 4H), 1.56−1.72 (m, 5H), 2.59 (s, 2H), 3.22 (d, 1H, J = 7.9 Hz), 7.08 (m, 1H), 7.19 (m, 1H), 7.40 (m, 2H); HRMS calcd for $C_{21}H_{28}O_3 + H_1$ 329.2117, found 329.2112. Anal. ($C_{21}H_{28}O_3 \cdot 0.27C_4H_{10}O$) C, H.

3-(α-Cyclopentylphenylmethyl)-5,6-dihydro-6,6-dipropyl-4-hydroxy-2*H*-pyran-2-one (XX): ^{1}H NMR ($CDCl_3$) δ 0.87 (m, 6H), 1.04 (m, 1H), 1.26 (m, 6H), 1.49−1.73 (m, 12H), 2.42 (d, 1H, J = 17.1 Hz), 2.47 (d, 1H, J = 17.1 Hz), 3.16 (m, 1H), 3.81 (d, 1H, J = 11.6 Hz), 7.09−7.49 (m, 5H); HRMS calcd for $C_{23}H_{32}O_3 + H_1$ 357.2430, found 357.2460.

Table 5. Summary of Selected Diffraction Data Collection and Refinement Statistics for the HIV Protease/Inhibitor Complexes

inhibitor complex	A	B	C	D	E
proteases	HIV-2	HIV-2	HIV-1[b]	HIV-1	HIV-2
inhibitors	**VI**	**VII**	**XII**	**XVII**	**XXIII**
space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12$	$P6_122$	$P2_1$[a]
unit cell *a*, Å	33.38	33.77	59.79	63.35	58.81
b, Å	45.49	46.07	87.57	63.35	44.10
c, Å	135.17	136.62	46.36	83.98	39.80
resolution, Å	2.4	2.6	2.2	2.5	2.2
no. of observations	25163	22872	53117	26326	19805
unique reflections	6998	5926	11686	3520	9623
completeness, %	80.5	83.3	90.6	93.0	94.0
R-merge, %	9.3	9.17	8.7	15.6	8.72
reflns in refinement	6840	5773	10472	3441	7811
R-refinement, %	17.3	15.3	19.3	18.3	17.4
rms deviations					
distance, Å	0.015	0.016	0.018	0.017	0.016
angle, deg	2.517	2.590	2.649	2.445	2.505
fixed dihedrals, deg	6.469	8.249	4.552	8.232	4.376
flex dihedrals, deg	15.598	15.707	15.224	15.434	15.062

[a] β, the monoclinic angle, in this case is 106.6°. [b] Triple mutant (Q7K/L33I/L63I).

Crystallography. Crystallization. The preparation and purification of the recombinant wildtype and the triple mutant (Q7K/L33I/L63I) of HIV-1 and HIV-2 proteases have been described elsewhere.[33−35] The protein preparation of HIV-2 protease contains a Lys57/Leu mutation but has been found to be indistinguishable in activity and specificity from the wildtype enzyme. The crystals of the proteases complexed with the selected inhibitors were obtained by cocrystallization experiments in which 2−5 μL of the inhibitor solution (of 0.1 mg/μL concentration) in DMSO was added to about 130 μL of the freshly thawed ice-cold protease solution (~6 mg/mL concentration), and the mixture was equilibrated on ice for about 1 h. The undissolved inhibitor that precipitated upon mixing was removed by centrifugation. Crystals were grown at room temperature in 10 μL hanging drops of equal volumes of protein/inhibitor complex and the precipitant from the well. In the case of HIV-1 protease, 0.75, 1.0, 1.5, and 2.0 M NaCl served as precipitants at pH's 4.8, 5.0, and 5.2 (0.1 M acetate buffer) and at pH's 5.4, 5.6, and 5.8 (0.1 M citrate buffer), and in the case of HIV-2 protease, 15, 20, 25, and 30% (w/v) PEG 4000 served as precipitants at pH's 6.6, 6.8, and 7.0 (0.1 M imidazole) and at pH's 7.2, 7.4, and 7.6 (0.1 M HEPES buffer). Best quality crystals were chosen out of these conditions, invariably from all of which we could obtain crystals.

Data Collection. A single crystal of each complex was used for data collection in all five cases. Diffraction data were collected using a Siemens area detector, with X-rays generated by a Siemens rotating anode source operating at 45 kV, 96 mA. Measurements were made as a series of 0.25° frames, with exposure times of 300 (for A, B, E), 180 (for C), and 240 (for D) s/frame. Data sets were processed using XENGEN data reduction software.[36] Table 5 summarizes the data collection statistics of the data sets along with statistics from the refinement of the models. The effective resolution of each crystal was taken as the maximum resolution for which the mean I/σ was greater than 2.0. Data beyond this maximum were discarded and not used in the crystallographic refinement.

Structure Refinement. Since the space groups of the protease/inhibitor complexes were the same as ones previously refined in our laboratory, refinement of these protease models could be initiated without having to resolve the position of the

asymmetric units in the respective unit cells. The structural refinement for the complexes was carried out using CEDAR[37] or X-PLOR[38] with periodic manual rebuilding using the iteractive graphics program CHAIN,[39] based on $2|F_o| - |F_c|$ and $|F_o| - |F_c|$ electron density maps. Electron density maps were calculated using the XTAL package of crystallographic programs.[40] The inhibitors and solvent molecules were added during later stages of the refinements. The atomic coordinates of these structures have been deposited in the Brookhaven Protein Data Bank.[41]

HIV-1 Protease Inhibitory Assay and K_i Value Determination. HIV-1 protease was purified and refolded from *Escherichia coli* inclusion bodies.[33] The substrate[42] used spans the p17−p24 processing site (R-V-S-Q-N-Y-P-I-V-Q-N-K) and was derivatized with biotin and fluorescein isothiocyanate at the amino and carboxy termini, respectively. The reaction was performed in assay buffer at substrate concentrations below the K_m concentrations for 90 min at room temperature in the dark. The assay buffer consisted of 0.1 M sodium acetate, 1.0 M NaCl, 0.05% NP40. Enzyme and substrate concentrations were 10 and 50 nM, respectively. Inhibitors were dissolved in dimethyl sulfoxide, and the amount in the assay was 2%. After incubation the reaction was stopped by addition of fluoricon avidin beads at 0.5% (w/v). The residual bound fluorescence was obtained by processing on an IDEXX screen machine from which percent inhibition values were calculated. A range of inhibitor concentrations was used. Determination of K_i values with this assay required analysis under conditions in which substrate concentration resided substantially below K_m and the inhibitor concentrations greatly exceeded the K_i value and the enzyme concentration. One can solve the remaining substrate after prolonged incubation times as a quadratic, exponential function in terms of inhibitor concentrations and time to generate calculated values for the kinetic constants K_m, k_{cat}, and K_i as well as the enzyme concentration.

Under these conditions, from measurement of the substrate (S) remaining at the end of a fixed time (t_f) in the presence of HIV protease enzyme (E_o) with each assay point one of a multitude of inhibitor concentrations (I), the following equation can be derived.

$$S = S_o \times e^{-\frac{k_{cat}t_f}{2K_m}\times(-I+E_o-K_i+\sqrt{(I-E_o+K_j)^2+4K_iE_o})} \quad (1)$$

where: S = substrate concentration, S_o = starting substrate concentration, E_o = analytical enzyme concentration, I = inhibitor concentration, k_{cat} = turnover constant for the catalytic reaction, K_m = Michaelis constant, and K_i = inhibitor constant, the dissociation constant of the enzyme/inhibitor complex, EI, according to the equation $K_i = E{\cdot}I/EI$, where E_o is the concentration of free enzyme.

The data are fit using a nonlinear least-squares analysis with the independent variable being the inhibitor concentration and the dependent variable being the substrate. The values for $k_{cat}/2K_m$, K_i, E_o, and S_o can be calculated from the regression analysis along with calculated residuals and a statistical evaluation of the how well the data fit the theoretical curve.

MTT Cytotoxicity Assay.[43] Into appropriate wells of a 96-well microtiter plate was added 125 μL of 2× drug dissolved in RPMI complete medium followed by 125 μL of suspension of MT4 cells containing 2×10^4 cells and mixed gently. Various drug concentrations were tested in triplicate. Positive controls contained cells and RPMI complete medium without drug, while negative controls contained medium only. Each microplate was incubated for 4−7 days at 37 °C, 5% CO_2. After completion of the incubation period, 100 μL of supernatant was removed, with minimal disturbance of the cells at the bottom of each well. To each well was added 10 μL of a freshly prepared MTT solution (5 mg/mL in PBS; Sigma). The plate was incubated for 4 h at 37 °C, 5% CO_2. An additional 50 μL was removed from all wells, and the cells were mixed thoroughly by vigorously pipetting up and down to resuspend the formazan dye precipitate. To each well was added 100 μL of 0.04 N HCl/2-propanol solution with extensive mixing. The plate was read within 15 min on a dual-wavelength microplate reader using a test wavelength of 570 nm and a reference wavelength of 650 nm. The cytotoxic activity of a test compound was calculated by dividing the mean OD at each drug concentration by the mean OD of the positive controls, subtracting the result from 1, and multiplying by 100. The $CCTD_{50}$ (cell culture toxicity dose$_{50}$) value was determined to be the calculated compound concentration required to inhibit 50% of cell metabolism when OD's between compound-exposed cells and compound-free control cells were compared. The $CCTD_{50}$ value was calculated by linear regression or straight line extrapolation.

Antiviral Activity (MT4/HIV-1_{IIIB} Assay).[44] MT4 cells were washed and resuspended in an inoculum of HIV-1_{IIIB} at a MOI of approximately 0.005. Adsorption occurred for 2 h at 37 °C, 5% CO_2. Unabsorbed virus was removed by low-speed centrifugation (200*g*, 10 min) of the cells followed by removal of the supernatant. The cell pellet was resuspended to a concentration of 8×10^5 cells/mL in RPMI complete medium. In appropriate wells of 24-well tissue culture plates (Corning no. 25820), 0.5 mL of infected MT4 cells and 0.5 mL of 2× test compound dissolved in RPMI complete medium plus 0.2% DMSO were combined. Each drug concentration was tested in triplicate. Virus control wells received RPMI complete medium plus 0.2% DMSO without compound. Final DMSO concentration in each culture was 0.1%. Plates were incubated for 7 days at 37 °C in 5% CO_2. At the end of the 7-day incubation period, 100 μL of supernatant was removed from each test well and live virus inactivated by the addition of lysis buffer (Coulter Diagnostics, Hialeah, FL) containing 5% Triton X-100. The amount of HIV p24 core antigen was quantified with an ELISA procedure by following the manufacturer's direction. The IC_{50} (inhibitory concentration$_{50}$), the amount of drug necessary to reduce the concentration of p24 in drug-containing cultures by 50% when compared to drug-free controls, was calculated by comparing the quantity of p24 produced in drug-containing supernatants with that of DMSO-containing drug-free supernatants. This was accomplished by using the linear portion of the plot of $\log_{10}$ drug concentration versus percent p24 inhibition and utilizing linear regression or straight line extrapolation to calculate the drug concentration necessary to inhibit 50% of non-drug-treated p24 antigen.

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