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Inhibition of the HIV-1 and HIV-2 Proteases by Curcumin and Curcumin Boron Complexes

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Abstract—Curcumin, a relatively non-toxic natural product isolated from *Curcuma longa*, is a modest inhibitor of the HIV-1 (IC_{50} = 100 μM) and HIV-2 (IC_{50} = 250 μM) proteases. Simple modifications of the curcumin structure raise the IC_{50} value but complexes of the central dihydroxy groups of curcumin with boron lower the IC_{50} to a value as low as 6 μM. The boron complexes are also time-dependent inactivators of the HIV proteases. The increased affinity of the boron complexes may reflect binding of the orthogonal domains of the inhibitor in intersecting sites within the substrate-binding cavity of the enzyme, while activation of the α,β-unsaturated carbonyl group of curcumin by chelation to boron probably accounts for time-dependent inhibition of the enzyme.

The proteases encoded by the HIV viral genomes are responsible for processing the polyprotein precursors produced from the *gag* and *pol* genes into the proteins required for replication and assembly of the mature virus.[1] Inactivation of the HIV-1 protease (HIV-1PR) by mutation of the catalytic aspartyl residues at positions 25 and 125 thus yields non-infectious virions.[2] The key role played by the protease and the availability of high resolution crystallographic structures for HIV-1PR,[3–5] have made this protease a highly attractive target for the design of anti-AIDS therapeutic agents.[6] The protease of the HIV-2 virus (HIV-2PR), which is widespread in West Africa, plays a similar role in replication of the virus but has been less amenable to direct structure-based inhibitor design due to the absence of a crystal structure for the enzyme. This deficiency has very recently been alleviated by determination of the crystal structures of HIV-2PR complexed with two peptide inhibitors.[7] Intensive efforts over the past five years have produced a variety of peptide inhibitors for both proteases with K_I values in the nanomolar to subnanomolar range, none of which has yet proven to be of clinical utility due to the low bioavailability and rapid degradation characteristic of peptidic agents. There is intense interest, therefore, in the development of non-peptidic inhibitors of the HIV proteases. In this context, we identified haloperidol, a clinically used antipsychotic agent, as an inhibitor of HIV-1PR with K_I = 100 μM.[8] Although structural modification has produced higher-affinity haloperidol analogues with IC_{50} values in the high nanomolar range, the toxicity of the haloperidol derivatives is still too high for the inhibitors to be of practical utility.[9] A crystal structure of the complex of HIV-1PR with the dithioketal derivative (1) of haloperidol shows that it is bound in an extended conformation in a site roughly orthogonal to, and above that, occupied by the peptide inhibitors for which crystal structures are currently available (Figure 1).[10] The flaps of the enzyme in the complex with 1 are in a semi-closed position intermediate between the open arrangement in the empty protein and the closed arrangement in complexes with peptide inhibitors. Compound 1 is bound in a different site when it is crystallized with a variant of HIV-1PR in which Glu-7 is replaced by a Lys and which is resistant to self-catalyzed proteolysis.[10,11] The flaps are fully closed in this second complex and the inhibitor is bound in the same general site as peptide inhibitors.[10] These structural studies indicate that there are at least two potential binding sites for non-peptidic inhibitors. The two sites are roughly orthogonal and at different distances from the catalytic aspartyl groups (Figure 1), so that both sites can, in principle, be simultaneously occupied.

Curcumin (2), a natural product of low toxicity isolated from the rhizomes of *Curcuma longa*, has been shown to have anti-inflammatory activity in both acute and chronic animal inflammation models. In animal studies, it is equipotent with phenylbutazone as an anti-inflammatory agent but is much less toxic. The oral LD_{50} in mice is greater than 2.0 g/kg.[12] We report here identification of curcumin as an inhibitor of HIV-1PR and HIV-2PR and a preliminary examination of the possibility of using structures with orthogonal domains as inhibitors of HIV-1PR and HIV-2PR.

Results

Curcumin

Curcumin was selected as a possible inhibitor of HIV-1PR for three reasons: (a) modeling studies indicated the curcumin structure fitted well into both of the potential binding sites in the crystal structure of HIV-1PR with 1, (b) the curcumin structure lends itself to the construction of molecules with orthogonal domains, and (c) curcumin itself is relatively non-toxic and, as suggested by its anti-inflammatory activity, is readily absorbed. The structure of curcumin used for the modeling studies was built and energy-minimized with SYBYL. The structure thus obtained was docked into the active site of HIV-1PR using the program DOCK 2.0 (single mode). The map, grid and

the sphere cluster of the enzyme were taken from the co-crystallized structure of HIV-1PR with **1**.[8] The computer docking experiments show that curcumin can bind in multiple sites, including both of the sites within the active site of HIV-1PR. The activity of curcumin as an inhibitor of HIV-1PR was examined *in vitro* using purified enzyme isolated from a heterologous *E. coli* expression system.[13] Comparative studies were carried out of the inhibition of HIV-2PR, for which no three dimensional structural coordinates were available. Curcumin is an inhibitor of HIV-1PR with IC_{50} = 100 μM (Table 1). Its activity is thus comparable to that of haloperidol.

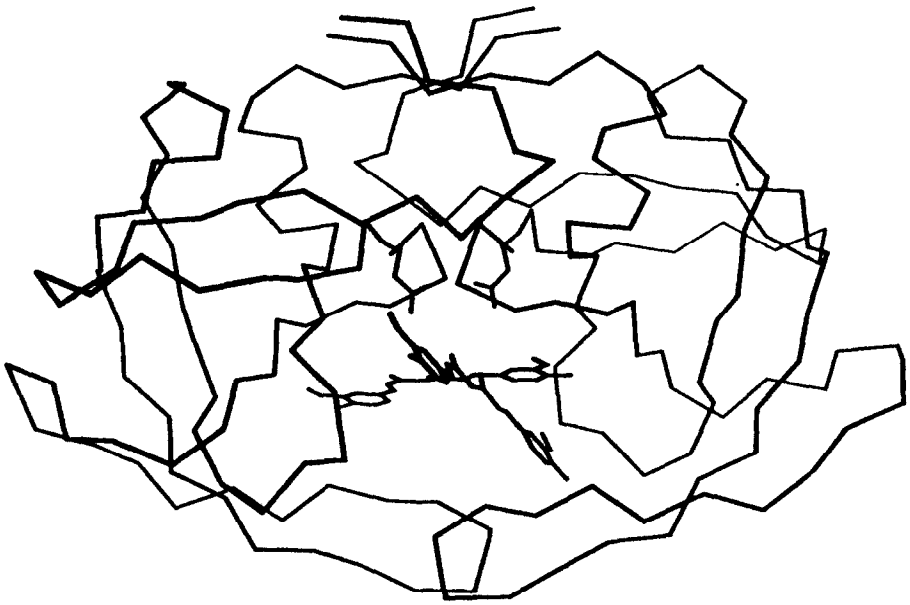
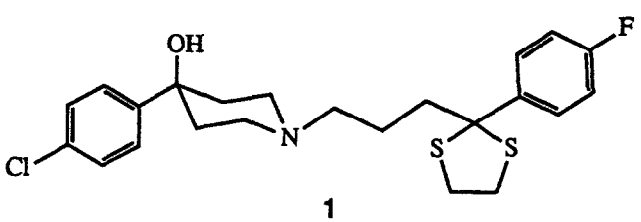


Figure 1. *Orthogonal inhibitor binding sites in the active site of HIV-1PR.* The structure shows haloperidol analogue **1** bound in the two roughly orthogonal orientations identified by X-ray crystallography in the active site of HIV-1PR[10]

Table 1. Inhibitory activities of curcumin and its derivatives against HIV-1PR

Compound	IC_{50} (μM)		Irreversible	Compound	IC_{50} (μM)		Irreversible
	HIV-1	HIV-2			HIV-1	HIV-2	
2	100	250	no	**9**	36	65	yes
3[a]	>50	>250	no	**10**	32	70	yes
4	>100	300	no	**11**	6	5.5	yes
5	>100	600	no	**12**	inactive		-
6	>100		no	**13**	inactive		-
7	24		yes	**14**	inactive		-
8	28	20	yes	**15**	inactive		-

[a]Insoluble at higher concentrations.



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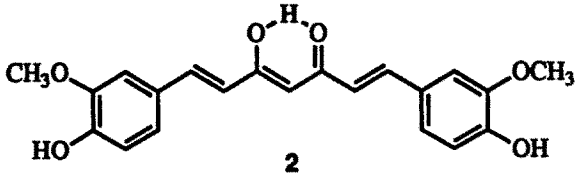

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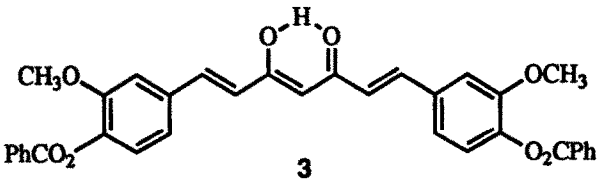

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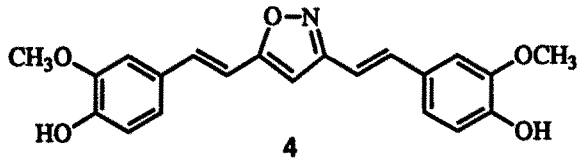

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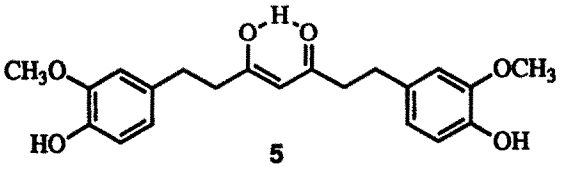

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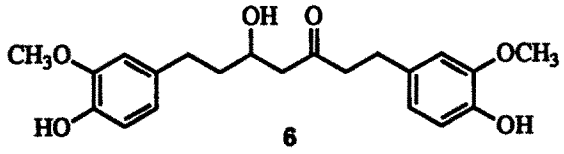

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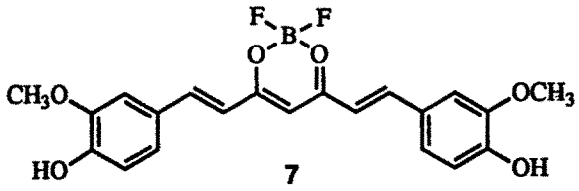

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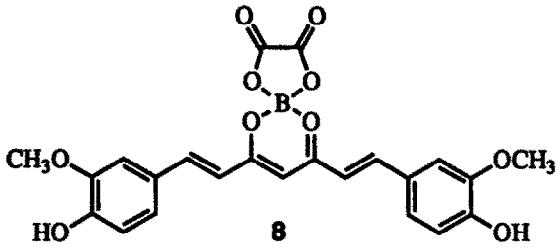

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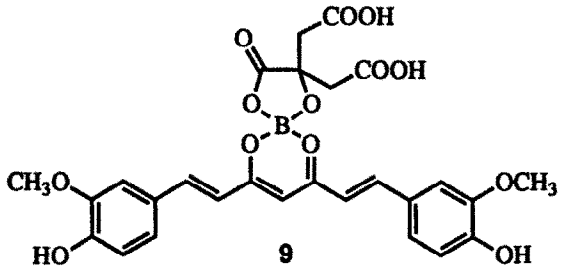

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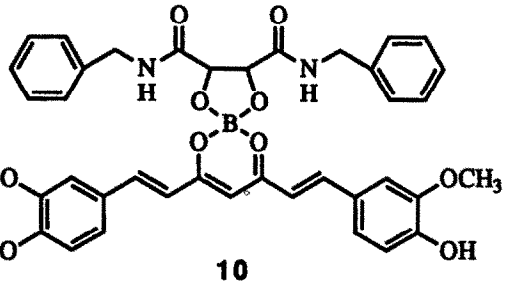

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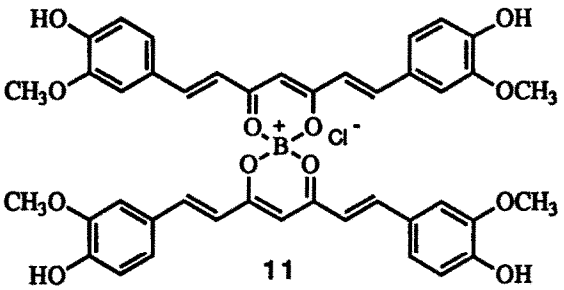

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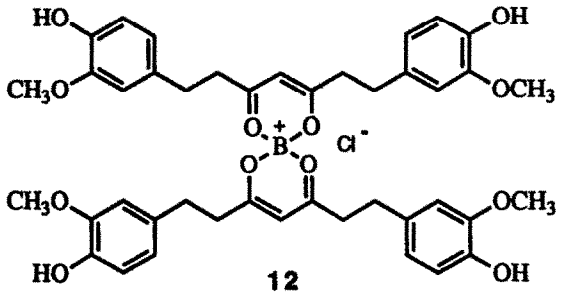

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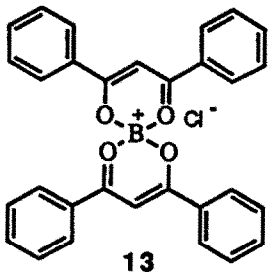

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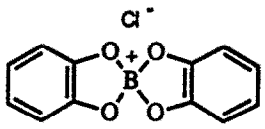

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Simple curcumin analogues

Simple modifications of the curcumin structure were examined to identify structural features essential for HIV-1PR inhibitory activity. Benzoylation of the phenolic groups, which yields compound **3**, and formation of the isoxazole **4** by reaction of the carbonyl groups with hydroxylamine, decreases inhibition of both HIV-1PR and HIV-2PR (Table 1). Reduction of curcumin with Raney nickel provides the tetra- and hexahydrogenated compounds **5** and **6**, respectively, both of which are less active than the parent compound. These results suggest that the phenolic hydroxyls are not essential for inhibition, whereas the planar network of conjugated double bonds connecting the two aromatic rings contributes significantly to inhibitory activity.

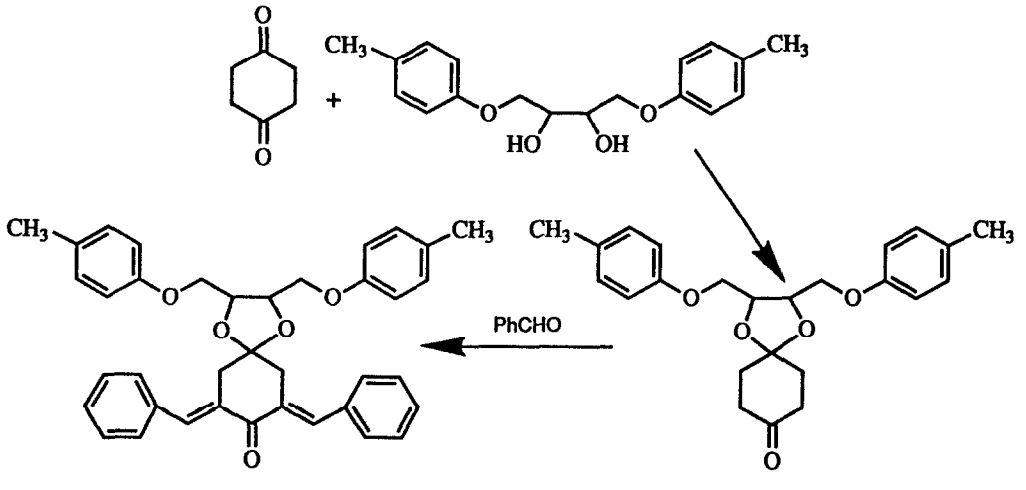
Curcumin boron complexes

Boron complexes of curcumin were prepared because the tetrahedral geometry about the boron atom forces two molecules, each of which provides two of the oxygen ligands, to assume an orthogonal orientation. Furthermore, boron complexes are readily formed with oxygen ligands and are relatively stable. The simplest complex examined was the difluoroboron complex **7**, in which two of the boron sites are occupied by fluorine atoms rather than by a second organic structure. The two fluorine atoms are replaced in mixed complexes by the carboxyl oxygens of oxalic acid (**8**), a carboxyl and hydroxyl group of citric acid (**9**), the two hydroxyl groups of dibenzyl tartramide (**10**), and a second molecule of curcumin (**11**). Compounds **10** and **11** are of particular interest because they consist of perpendicular domains, each of which is an extended organic structure with aromatic rings at the termini. In fact, all of the mixed boron complexes that contain at least one curcumin unit are more potent inhibitors of both HIV proteases than curcumin itself (Table 1). The best of the boron complexes is **11**, which gives IC_{50} values of 6 and 5.5 μM for HIV-1PR and HIV-2PR, respectively (Table 1).

To determine whether the boron component plays an independent role in inhibition of HIV-1PR, compounds **12–14**, each of which contains a tetrahedral borate ester center but no curcumin moiety, were examined. None of these compounds has detectable inhibitory activity. These results suggest that the curcumin moiety is essential for the biological activity of the boron complexes. In a further experiment to determine the role of the boron, an analogue (**15**) was synthesized (Scheme I) in which the boron center of the molecule was replaced by a spiro carbon atom. Compound **15** has a geometry similar to that of **10** and **11**, although the two orthogonal substructures differ from those found in compounds **10** and **11**. Compound **15** has no HIV-1PR inhibitory activity, however. It is not possible to determine from the available data whether the inactivity of **15** is due to a specific role of the boron in complexes **10** and **11**, although the inactivity of complexes **12–14** indicates that a boron core is not sufficient for inhibition of the enzyme. The differences in the structures of the orthogonal domains of **15** versus **10** and **11** presumably contributes to the marked differences in the activities of these compounds.

Time-dependent inhibition of the protease

Time-dependence studies suggest that compounds **7–11** are irreversible inhibitors of HIV-1PR and HIV-2PR (Figure 2). None of the other compounds is a detectable time-dependent inhibitor of these enzymes. The parameters for inactivation of HIV-1PR by **11**, the most effective compound, are k_{inact} = 468.3 ± 67.5 μM and V_{inact} = 0.041 ± 0.006 min^{-1} (Figure 2). The corresponding values for inactivation of HIV-2PR by **11** are k_{inact} = 260.7 ± 24.7 μM and V_{inact} = 0.022 ± 0.002 min^{-1} (Figure 2). Although the mechanism of this irreversible process has not been determined, it is probable that the α,β-unsaturated ketone substructure in the curcumin moiety of **7–11** acts as a Michael acceptor. The intrinsic reactivity of the α,β-unsaturated ketone in these compounds is enhanced by coordination of the ketone group to boron. The nucleophile on the enzyme that is involved in the inactivation reaction has not been specifically identified, but studies of irreversible inhibitors based on the haloperidol structure indicate that the likely sites in HIV-1PR are the cysteine sulfhydryl groups at positions 67, 95, 167, and 195 or the catalytic aspartyl groups at positions 25 and 125.[9,14] In HIV-2PR, which has no sulfhydryl groups, the most susceptible residues are the two catalytic aspartyl groups.



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Scheme I. Synthetic scheme for the preparation of compound **15**.

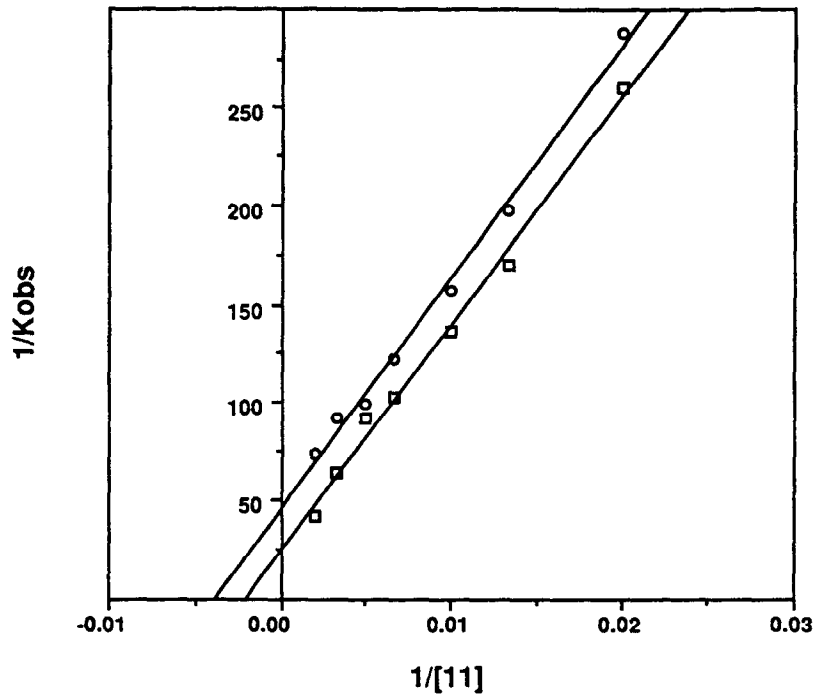

Figure 2. *Irreversible inhibition of HIV-1PR by compound 11.* Inactivation of HIV-1PR (□) and HIV-2PR (○) by **11** has been determined as described in the Experimental Section. Experimental data has been fitted according to the equation: $Ln(v_i/v_0) = -K_{obs}*t$. From a double reciprocal plot of inactivation rates (k_{obs}) versus inhibitor concentrations the K_{inact}, inhibitor concentration resulting in half-maximal inactivation and the maximum inactivation rate, V_{inact}, were calculated

Conclusions

The modest activity of curcumin as an inhibitor of HIV-1PR and HIV-2PR is enhanced more than 10-fold when curcumin dimer **11** is formed by coordinating the central keto-enol groups of the two curcumin units to a boron atom. The activity of dimer **11** is 4–6 times greater than the activity of complexes in which curcumin is coordinated to a boron difluoride moiety (**7**) or to boron with small dioxygen ligands occupying the other two boron coordination sites (e.g. **8–10**). Complexation with boron therefore does not account for the higher inhibitory activity of **11**. It is therefore possible that the higher affinity of **11** is due to simultaneous occupation of two binding sites in the proteases, although the decreases in the IC_{50} values are lower than might be expected for simultaneous occupancy of two binding sites. One possible explanation for this is that the inhibitor domains are truly orthogonal, whereas they are not truly orthogonal in the enzyme active sites (Figure 1). The inflexibility of the orientation of the two curcumin groups about the boron atom therefore may not permit optimal fit of the inhibitor in the substrate binding sites. On the other hand, binding to the boron activates the unsaturated carbonyl moiety of curcumin towards electrophilic additions and makes the curcumin complexes time-dependent inhibitors of the two enzymes. Although the nature of the time-dependent inhibition has not been precisely defined, it is likely that it involves alkylation of one of the two active site aspartyls and/or one of the four cysteines of HIV-1PR, and one of the two active site aspartyls in HIV-2PR, by the boron-activated α,β-unsaturated carbonyl structure in the inhibitors.[9,14]

Experimental Section

General

Melting points were determined with a Thomas capillary melting point apparatus and are uncorrected. ^1H and ^{13}C NMR spectra were obtained at 300 and 75 MHz, respectively, on a GE QE-300 instrument. Infrared spectra were determined on a Nicolet 5DX FT-IR. Mass spectra were measured with a VG-70 mass spectrometer interfaced with a Hewlett-Packard 5890A gas chromatograph. Elemental analyses were performed by the Microanalysis Laboratory, University of California, Berkeley. Dimethylformamide was dried over 4 Å molecular sieves, distilled at reduced pressure, and stored over 4 Å sieves under argon. Diisopropylethylamine and dichloromethane were heated at reflux over calcium hydride, distilled under argon, and stored over 4 Å sieves under argon. THF and ether were dried by refluxing over sodium. Curcumin (**1**) was purchased from Janssen/Spectrum (Gardena, CA).

Dibenzoyl curcumin (3)

A mixture of curcumin (368 mg, 1 mmol), benzoyl chloride (281 mg, 2 mmol) and dry pyridine (0.5 ml) in THF (10 ml) was stirred at ~25 °C for 24 h. Ethyl acetate was then added and the mixture was washed with 1 N HCl, brine, saturated $NaHCO_3$ solution, and brine before it was dried over anhydrous $MgSO_4$. The residue obtained upon evaporation of the solvent was recrystallized from acetic acid. Compound **3** (502 mg, 87%) was obtained as a yellow solid, m.p. 175 °C (lit.[15] 176–178 °C): ^1H NMR

(300 MHz, $CDCl_3$) 3.85 (s, 6H, OCH_3), 5.86 (s, 1H, CH), 6.65 (d, 2H, =CH), 7.22 (m, 4H, Ar CH–CH), 7.52 (t, 4H, *m*-H in PhCOO), 7.65 (m, 2H, *p*-H in PhCOO), 7.67 (d, 2H, =CH), and 8.23 ppm (d, 4H, *o*-H in PhCOO); EIMS m/z 576 (16), 574 (25), 105 (100). HRMS calcd for $C_{25}H_{28}O_8$: 576.1784; found 576.1757.

Isoxazolcurcumin (4)

A mixture of curcumin (100 mg, 0.27 mmol) and hydroxylamine hydrochloride (200 mg, 2.88 mmol) in ethanol (20 ml) was heated under reflux for 16 h. Water and ethyl acetate were then added and the organic phase was washed with brine and dried over anhydrous $MgSO_4$. After evaporation of the solvent, the residue was purified by silica gel chromatography (1/1 hexane/ethyl acetate). Compound **4** (68 mg, 69%) was obtained as a white solid, m.p. 172 °C (lit.[16] 173 °C): ^1H NMR (300 MHz, $CDCl_3$) 3.90 (s, 3H, OCH_3), 3.91 (s, 3H, OCH_3), 6.71 (s, 1H, isoxazole CH), 6.84–7.33 (3m, 2, 4, 4H, Ar and =CH), and 7.94, 8.01 ppm (2s, 2H, OH); ^{13}C NMR (75 MHz, $CDCl_3$) 56.3, 98.4, 110.2, 110.6, 111.4, 114.0, 115.9, 116.1, 116.5, 122.3, 128.7, 129.1, 129.4, 129.6, 135.5, 136.8, 148.7, 163.1, and 169.5 ppm; EIMS m/z 365 (100), 336 (54), 306 (23), 229 (35), 177 (30). HRMS calcd for $C_{21}H_{19}O_8$: 365.1263; found 365.1250.

Hydrogenation of curcumin (5 and 6)

Raney-Ni (~ 30 mg, 50% slurry in H_2O, pore size 50 μ, surface area 80–100 m^2/g) was added to a solution of curcumin (368 mg, 1 mmol) in a mixture of THF (4 ml) and methanol (1.5 ml) in a hydrogenation flask. Hydrogen was passed through the mixture and the mixture was shaken at 20 psi in a Parr hydrogenator for 4 h. The mixture was then filtered through celite, the solvent was removed under vacuum, and the residue was separated by silica gel column chromatography (1/1/0.01 hexane:ethyl acetate:acetic acid). Two fractions were collected as oils: fraction I, R_f = 0.6 and fraction II, R_f = 0.2. Fraction I was identified as tetrahydrocurcumin (**5**): ^1H NMR (300 MHz, $CDCl_3$) 2.55 (t, J = 7 Hz, 4H, CH_2Ar), 2.75 (t, J = 7 Hz, 4H, CH_2CO), 3.75 (s, 6H, OCH_3), 5.45 (s, 1H, CH=), 6.61–6.64 (m, 4H, aromatic), and 6.80 ppm (d, J = 8 Hz, 2H, aromatic); ^{13}C NMR (75 MHz, $CDCl_3$) 31.2, 40.2, 55.7, 99.8, 110.9, 114.3, 120.7, 132.4, 143.8, 146.4, and 193.4 ppm. HRMS: calcd 372.1573; found 372.1591. Fraction II was identified as hexahydrocurcumin (**6**):[17] ^1H NMR (300 MHz, $CDCl_3$) 1.62–1.79 (m, 2H, $HOCHC\underline{H}_2$), 2.52–2.59 (m, 4H, $C\underline{H}_2$–Ar), 2.70 (t, 2H, J = 7 Hz, $CH_2C\underline{H}_2CO$), 3.81, 3.82 (2s, 6H, OCH_3), 4.04–4.08 (m, 1H, C<u>H</u>OH), 7.33 (d, J = 9 Hz, 2H, aromatic), 7.44 (s, 2H, aromatic), 6.61–6.68 (m, 4H, aromatic), and 6.80 ppm (d, J = 8 Hz, 2H, aromatic); ^{13}C NMR (75 MHz, $CDCl_3$) 29.1, 31.2, 38.1, 45.2, 49.1, 55.7, 67.0, 111.0, 111.1, 114.3, 114.4, 120.6, 120.7, 132.4, 133.5, 143.5, 143.8, 146.4, and 211.5 ppm; EIMS m/z 372 (M^+, 27), 342 (M-2CH_3, 13), and 137 (100). HRMS: calcd 374.1729; found 374.1723.

Difluoroboron complex of curcumin (7)

Boron trifluoride etherate (62 ml, 0.5 mmol) was added at ~25 °C to a solution of curcumin (184 mg, 0.5 mmol) in 5 ml of dry THF. The mixture was stirred for 16 h at ~25 °C before the solvent was evaporated and the residue was recrystallized from ethyl acetate and dichloromethane. Compound **7** was obtained as violet needles: UV λ_{max} = 498 nm; ^1H NMR (300 MHz, $CDCl_3$) 3.93 (s, 6H, OCH_3), 6.33 (s, 1H, CH=), 6.88–6.91 (2d, 4H, CH=/aromatic), 7.33 (d, J = 9 Hz, 2H, aromatic), 7.44 (s, 2H, aromatic), 7.93 (d, J = 15 Hz, 2H, CH=), and 8.51 ppm (s, 2H, OH); ^{13}C NMR (75 MHz, $CDCl_3$) 56.3, 101.9, 112.5, 116.5, 119.0, 125.7, 127.6, 147.5, 148.9, and 151.8 ppm. LSIMS m/z 416 (M^+, 7), 397 (M^+ - F, 100).

Boron complex of curcumin with oxalic acid (8)

A suspension of curcumin (369 mg, 1 mmol), boric acid (61.8 mg, 1 mmol) and oxalic acid dihydrate (126 mg, 1 mmol) in toluene was heated under reflux using a Dean–Stark trap for 16 h. After filtration the solid was recrystallized from ethyl acetate and acetonitrile. Compound **8** was obtained as a black solid (350 mg, 75%): UV λ_{max} = 532 nm; ^1H NMR (300 MHz, acetone-d_6) 3.84 (s, 6H, OCH_3), 6.60 (s, 1H, CH), 6.88, 7.37 (2d, J = 8 Hz, 4H, Ar), 7.13, 8.04 (2d, J = 16 Hz, 4H, CH=CH), 7.48 (s, 2H, Ar), and 10.25 ppm (s, br, 2H, OH); LSIMS m/z 467 (40, M + 1).

Boron complex of curcumin with citric acid (9)

The title compound was obtained as a purple solid (310 mg, 54%) from curcumin (369 mg, 1 mmol), boric acid (61.8 mg, 1 mmol) and citric acid (192 mg, 1 mmol) by the procedure reported for the preparation of **8**: UV λ_{max} = 496 nm; ^1H NMR (300 MHz, acetone-d_6) 2.75, 2.77 (2s, 4H, CH_2), 3.83, 3.86 (2s, 6H, OCH_3), 6.41 (s, 1H, CH), 6.88, 7.28 (2d, J = 8 Hz, 4H, Ar), 6.96, 7.86 (2d, J = 16 Hz, 4H, CH=CH), and 7.42 ppm (s, 2H, Ar); LSIMS m/z 569 (M^+ + 1).

Boron complex of curcumin and N,N'*-dibenzyl-D-tartramide (10)*

A suspension of curcumin (184 mg, 0.5 mmol), boric acid (31 mg, 0.5 mmol) and *N,N'*-dibenzyl-D-tartramide (164 mg, 0.5 mmol) in toluene (20 ml) was heated under reflux using a Dean–Stark trap for 24 h. The solid obtained by filtration was recrystallized from THF. Compound **10** was obtained as a dark violet solid (201 mg, 56%, impure): ^1H NMR (300 MHz, acetone-d_6) 3.84 (s, 6H, OCH_3), 4.52–4.67 (m, 4H, $C\underline{H}_2Ph$), 4.80 (s, 2H, >C<u>H</u>–O-), 6.30 (s, 1H, CH), 6.75–7.02 and 7.25–7.45 ppm (2m, 20H, Ar, CH=CH); LSIMS m/z 705 (29, M^+ + 1), 675 [(M^+ + 1) - 2 CH_3].

Curcumin–boron-complex chloride (11)

Boron trichloride (0.5 mmol, 1.0 M in CH_2Cl_2) was added at -78 °C to a solution of curcumin (369 mg, 1 mmol) in a mixture of ether (20 ml) and methanol (10 ml). The temperature was allowed to rise to 0 °C and the mixture was stirred for 1 h. The solvent was removed under vacuum and the residue was recrystallized from methanol. TLC and NMR showed that the deep purple solid (305 mg), m.p. >300 °C, was a mixture of **11** and curcumin: UV λ_{max} = 540 nm; LSIMS m/z 745 (M^+, 20), 715 (17).

Boron complex of tetrahydrocurcumin (12)

Boron trichloride (1 M in CH_2Cl_2, 0.108 mmol) was added to a solution of **5** (80 mg, 0.215 mmol) in dry dichloromethane (5 ml). The mixture was stirred at ~25 °C for 2 h. The precipitate was then filtered and recrystallized from acetonitrile. Compound **12** was obtained as a white solid: ^1H NMR (300 MHz, DMSO-d_6) 2.55 (t, J = 7 Hz, 4H, CH_2Ar), 2.78 (t, J = 7 Hz, 4H, CH_2CO), 3.73, 3.75 (2s, 6H, OCH_3), 5.73, 5.75 (2s, 2H, CH=), and 6.55–6.82 ppm (m, 6H, aromatic); ^{13}C NMR (75 MHz, DMSO-d_6) 28.4, 30.5, 55.5, 99.7, 112.4, 115.3, 120.2, 129.1, 131.3, 144.9, 147.4, and 193.4 ppm; LSIMS m/z 753 (M^+, 22), 723 ($M^+ - 2CH_3$, 12).

Boron complex of dibenzoylmethane 13[18]

Boron trichloride (2.3 mmol, 1.0 M in CH_2Cl_2) was added at 0 °C to a solution of dibenzoylmethane (1 g, 4.46 mmol) in dichloromethane (2 ml). The mixture was stirred at ~25 °C for 10 min and refluxed for 10 min. After cooling, the precipitate was filtered, washed with dichloromethane, and recrystallized from acetonitrile. Compound **13** was obtained as a slightly yellow solid (0.9 g, 82%): IR (NaCl) 1553, 1483, 1349, and 1103 cm^{-1}; ^{13}C NMR (75 MHz, acetone-d_6) 93.2, 127.4, 128.9, 133.0, 134.6, and 185.3 ppm; LSIMS m/z 457 (M^+ + 1).

Compound 14[19]

A solution of catechol (2.2 g, 20 mmol) and boric acid (0.62 g, 10 mmol) in methanol (15 ml) was heated under reflux for 17 h. Water and potassium carbonate (1.38 g, 10 mmol) were added to the cooled reaction mixture. The precipitate was filtered, washed with water (2x), and dried under vacuum. Compound **14** was obtained as a white solid (1.8 g, 68%), m.p. >250 °C: IR (NaCl) 1595, 1490, 1251, 1209, 1117, and 950 cm^{-1}; ^1H NMR (300 MHz, acetone-d_6) 6.47 ppm (m, Ar); ^{13}C NMR (75 MHz, acetone-d_6) 107.60, 117.29, and 151.53 ppm.

Spiro carbon analogue 15

A solution of 1,4-cyclohexadione (104 mg, 0.465 mmol), D-threitol 1,4-di-*p*-tosylate (200 mg, 0.465 mmol) and *p*-toluenesulfonic acid (5 mg) in toluene (20 ml) was heated under reflux with a Dean–Stark trap for 24 h. The cooled mixture was washed with $NaHCO_3$ solution and brine before it was dried over anhydrous $MgSO_4$. Solvent removal yielded a residue that was purified by silica gel column chromatography (1/1 hexane:ethyl acetate). The mono-ketal obtained by condensation of D-threitol 1,4-di-*p*-tosylate with 1,4-cyclohexadione (73.4 mg, 30%) was obtained as a colorless oil: ^1H NMR (300 MHz, $CDCl_3$) 1.92 (t, J = 7 Hz, 4H, $CH_2C<$), 2.39 (t, J = 7 Hz, 4H, CH_2CO), 2.45 (s, 6H, CH_3), 4.14–4.16 (m, 6H, OCH, OCH_2), 7.36 and 7.75 ppm (2d, J = 8 Hz, 8H, aromatic); ^{13}C NMR (75 MHz, $CDCl_3$) 21.5, 34.6, 37.7, 67.9, 74.9, 109.2, 127.7, 130.0, 132.3, 145.3, and 209.3 ppm; LSIMS m/z 525 (M^+ + 1, 100).

A solution of the mono-ketal (36 mg, 0.069 mmol) obtained by condensation of D-threitol 1,4-di-*p*-tosylate with 1,4-cyclohexadione, benzaldehyde (15 mg, 0.14 mmol) and sodium methoxide (7.5 mg, 0.14 mmol) in methanol (2 ml) was heated under reflux for 16 h. Ethyl acetate was added to the cooled mixture. The organic phase was washed with brine and dried over anhydrous $MgSO_4$. Purification of the residue obtained by silica gel column chromatography (1/1 hexane:ethyl acetate) yielded compound **15** (15 mg, 31%) as a colorless oil: ^1H NMR (300 MHz, $CDCl_3$) 2.42 (s, 6H, CH_3), 3.18 (s, 4H, CH_2 in ring), 3.99–4.20 (m, 6H, OCH, OCH_2), 7.26–7.66 (m, 18H, aromatic), and 7.85 ppm (s, 2H, =CH); ^{13}C NMR (75 MHz, $CDCl_3$) 21.7, 38.5, 67.7, 75.2, 127.9, 128.5, 128.7, 129.9, 130.0, 130.3, 131.4, 135.2, 139.4, 145.3, and 187.7 ppm. LSIMS m/z 701 (100, M + 1).

In vitro *assay of HIV PR inhibition*

Recombinant HIV-1PR was expressed in *E. coli* strain D1210 using the pSOD/PR179 vector.[13] Recombinant HIV-2PR was expressed in *E. coli* strain X90 using the pT2HIV2/115 vector.[11] The enzyme was purified to homogeneity, as judged by a single band on a silver stained sodium dodecyl sulfate polyacrylamide gel, using a combination of ion-exchange chromatography and affinity chromatography on pepstatin-A agarose. Concentrations of active HIV PRs were determined by active site titration using the peptidomimetic inhibitor U-85548 (a gift from Dr A. Tomasselli, Upjohn), Val-Ser-Gln-Asn-Leu-Ψ[CH(OH)CH_2]-Val-Ile-Val.[20]

HIV-1PR and HIV-2PR were assayed against the decapeptide Ala-Thr-Leu-Asn-Phe-Pro-Ile-Ser-Pro-Trp, which corresponds to the HIV-1PR C-terminal auto-processing site. The decapeptide was synthesized by conventional solid-state methods. Reactions were carried out as described.[8] Conversion of the decapeptide to the two pentapeptides was quantitated by integration of the peak areas after separation on HPLC and comparison with product standard curves. The IC_{50} determinations were carried out at pH 5.5. Stock solutions (1 μM–50 mM) of the inhibitors were prepared in DMSO. Compounds were added to assay solutions containing additional DMSO to give a final concentration of 5% (vol/vol). Baseline values were determined from enzymatic reactions containing 5% DMSO with no inhibitor present. HIV-1PR and HIV-2PR were preincubated with the different inhibitors for 5 min at 25 °C, followed by addition of the decapeptide substrate (250 μM final concentration) to initiate the reaction.

Inactivation of HIV PRs

To quantify irreversible inactivation of the HIV proteases by haloperidol derivatives, HIV-1PR (15 μg/ml final concentration) and HIV-2PR (30 μg/ml final concentration) were preincubated at 25 °C in 50 mM Hepes pH 8.0 buffer containing 0.1 M NaCl, 1 mM EDTA, 500 μM DTT, and 5% DMSO in the presence of 50–500 μM **11**. Baseline measurements were carried out using 5% DMSO in the absence of inhibitors. At various times, aliquots were removed and assayed for activity. HIV proteases were assayed against the fluorescent substrate ABZ-Thr-Ile-Nle-Phe(p-NO_2)-Gln-Arg-NH_2. The enzymes were assayed at pH 5.4 in 1 M NaCl, 1 mM EDTA, 1 mM DTT as previously described.[21] Kinetic data were fitted to a pseudo-first order equation to calculate the rates of enzyme inactivation.

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