Inhibition of the HIV-1 Protease by Fullerene Derivatives: Model Building Studies and Experimental Verification

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Abstract: The ability of C_{60} fullerene ("Bucky Ball") derivatives to interact with the active site of HIV-1 protease (HIVP) has been examined through model building and simple physical chemical analysis. The model complexes generated via the program DOCK3 suggest that C_{60} derivatives will fit snugly in the active site, thereby removing 298 Å^2 of primarily nonpolar surface from solvent exposure and driving ligand/protein association. The prediction that these compounds should bind to the active site and thereby act as inhibitors has been borne out by the experimental evidence. Kinetic analysis of HIVP in the presence of a water-soluble C_{60} derivative, bis(phenethylamino-succinate) C_{60}, suggests a competitive mode of inhibition. This is consistent with and supports the predicted binding mode. Diamino C_{60} has been proposed as a "second-generation" C_{60} derivative that will be able to form salt bridges with the catalytic aspartic acids in addition to van der Waals contacts with the nonpolar HIVP surface, thereby improving the binding relative to the tested compound.

Introduction

The protease specific to the human immunodeficiency virus 1 (HIVP) has been shown to be a viable target for antiviral therapy.[1] The active site of this enzyme can be roughly described as an open-ended cylinder which is lined almost exclusively by hydrophobic amino acids (Figure 1a). Notable exceptions to this hydrophobic trend are the two catalytic aspartic acids (Asp 25, Asp 125) which catalyze the attack of water on the scissile peptide bond of the substrate. We hypothesized that since a C_{60} molecule (i.e., fullerene) has approximately the same radius as the cylinder that describes the active site of the HIVP and since C_{60} (and its derivatives) is primarily hydrophobic, an opportunity therefore exists for a strong hydrophobic interaction between the C_{60} derivative and active site surfaces. This interaction should make C_{60} derivatives inhibitors of the HIVP. In this work, we describe model complexes of C_{60} and HIVP generated via the program DOCK3. The surface that is desolvated due to complex formation is shown to be almost exclusively hydrophobic. In addition, kinetic analysis is presented that supports a competitive mode of inhibition of a tested C_{60} derivative, consistent with our intuition and the complexes generated. Finally, we propose and validate the design of an amino-derivatized C_{60} as a reasonable next step in improving the binding energy of C_{60} derivatives to the HIVP.

Results and Discussion

To test the hypothesis regarding the complementarity of the C_{60} with the HIVP active site, a model of C_{60} was created and minimized using the SYBYL package (Version 5.4, Tripos Associates, Inc.). The model produced had a diameter within 0.2 Å of a spectroscopically determined C_{60} structure.[2] This model was fitted into the active site of the so-called "open" (i.e., uncomplexed) form of the HIVP[3] using the program DOCK3.[4] DOCK3 finds optimal orientations of a ligand with its receptor, scoring on the basis of van der Waals contacts and complementary electrostatics. This procedure produced complexes with the C_{60} squarely in the center of the active site, forming good van der Waals contacts with the active site surface, thereby reinforcing our model. Figures 1b and 1c show the highest scoring complex of C_{60} with HIVP in "front" and "side" views, which show the van der Waals surface contacts.

The change in solvent-exposed surface upon binding was determined in order to approximate the maximum magnitude of hydrophobic interactions. This was accomplished by first determining the total surface area of the active site and C_{60} molecules separately and then subtracting the total surface area of the highest scoring DOCK3 C_{60}/HIVP complex. All surface areas were determined from molecular surfaces generated by the program MS (Michael Connolly, University of California, San Francisco). The calculation indicates that 298 Å^2 of primarily hydrophobic surface is removed from solvent exposure. This total desolvated surface was further characterized by summing the individual surface elements according to atom type. The result of this summation (Table I) is that the large majority (273 Å^2 or 92%) of the desolvated surface is due to C_{60}-carbon/HIVP-carbon atom contact. The small amount of oxygen desolvation (7%) is due primarily to the partial blockage of the catalytic aspartates. Using the figure of 69.2 cal/(mol·Å^2) recently shown to accurately describe the free energy released upon desolvation of hydrophobic molecular surface,[5] we calculated the resultant free energy gain upon binding due to the carbon surface that is desolvated to be 19 kcal/mol. In order to estimate an approximate binding constant of a C_{60} derivative, this value has to be corrected for the free energy cost due to loss of translational/rotational entropy that accompanies binding. This value has been estimated to be on the order of 7–11 kcal/mol.[6] After we took this energetic cost into account, the result is a total ΔG_{bind} of 8–12 kcal/mol. Converting this to K_d values using the expression $\Delta G^\circ = -RT \ln K_d$ results in dissociation constants on the order of 10^{-6}–10^{-9} M. Several factors have been left out of this analysis, for example, rotational entropy persistence of the C_{60} in the active site, conformational energy of the HIVP, and interaction of the

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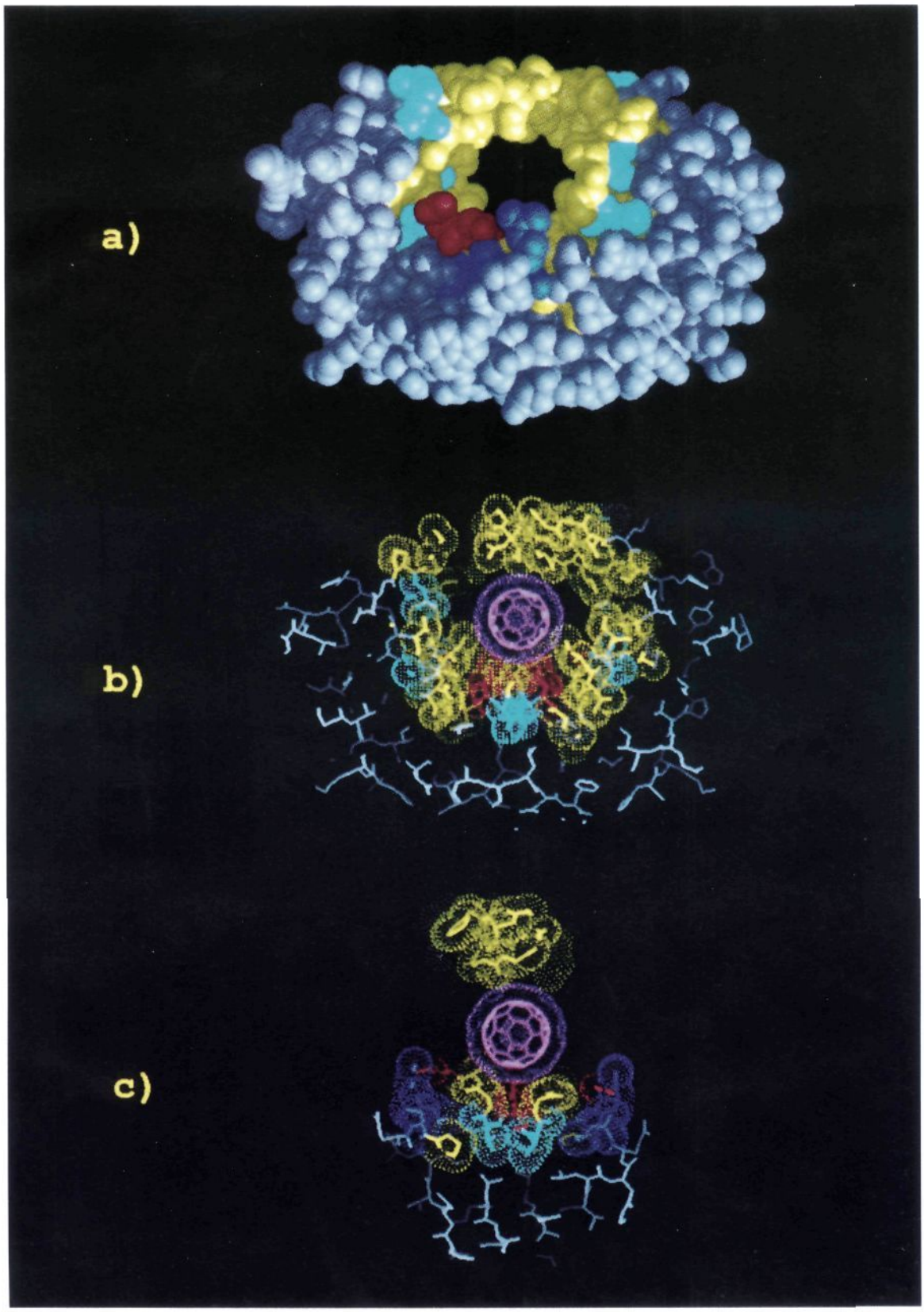


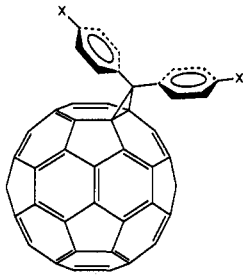
Figure 1. (a) "Front" view of the HIV-1 protease. Color coding is as follows. Yellow: Leu, Ile, Phe, Tyr, Val, Trp, Pro, Gly, Ala. Blue: Lys, Arg. Red: Asp, Glu. Cyan: Thr, Ser, Gln, Asn, Cys, Met, His. Gray: regions greater than 10 Å from the center of the active site. (b) Same view as (a) with the top scoring C_{60} orientation shown. The C_{60} is colored magenta, and the van der Waals surface of the active site and ligand are shown. (c) Same complex as (b) seen at a 90° cross section.

catalytic aspartates with the C_{60} surface. The purpose of this analysis is to account for the factors influencing binding that are reasonably estimated from our understanding of protein–ligand interactions.

Table I. Breakdown of Molecular Surface Changes upon C_{60}/HIVP Complexation According to Atom Type[a]

compd	C	N	O
complex (HIVP + C_{60})	1537.64	109.272	266.456
HIVP	1402.55	112.504	287.898
C_{60}	408.95	0	0
total change	-273.31	-3.232	-21.442

[a] The surface areas of the complex and of HIVP were determined for an identical subset of the total protein structure which contained and flanked the active site.



1, X = $HOC(O)(CH_2)_2C(O)NH(CH_2)_2$-

Figure 2. Compound 1.

A relatively synthetically accessible water-soluble C_{60} derivative, bis(phenethylamino-succinate) C_{60} (compound 1, Figure 2), was the first test of our hypothesis. The synthesis and characterization of this compound are described in the accompanying manuscript.[7] The highest scoring DOCK3 complex of this compound with the HIVP again positions the core C_{60} in the center of the active site, with the charged side chains extending through the mouth of the active site into solution (Figure 3). The ability of this compound to inhibit the HIVP was assayed with an HPLC method,[8] and its K_i value was found to be 5.3 μM (SE 0.98). The kinetic data fit the pattern of competitive inhibition well (Figure 4). This supports the proposed model complex, as the predicted binding mode of the C_{60} core should preclude any inhibitor binding while substrate is bound. It is of interest to note that compound 1 has been found to inhibit acutely and chronically HIV-1 infected human peripheral blood mononuclear cells (PBMC) with an EC_{50} of 7 μM while showing no cytotoxicity in uninfected PBMC (Raymond F. Shinazi et al., manuscript in preparation).

This introductory example demonstrates the potential for C_{60}-based inhibitors of the HIVP. As a point of comparison, the best peptide-based inhibitors are effective in the subnanomolar range and the best nonpeptide inhibitors are effective in the high nanomolar range.[9-11] The main driving force behind the association of the HIVP and the fullerene derivative examined is presumably hydrophobic interaction between the nonpolar active site surface and the C_{60} surface. In addition, however, there is an opportunity for increasing binding energy by the introduction of specific electrostatic interactions. An obvious possible electrostatic interaction is a salt bridge between the catalytic aspartates on the floor of the active site and a cationic site on the C_{60} surface. It has been found that several dicationic metals are effective inhibitors of the HIVP.[12,13] The authors of this work hypothesized

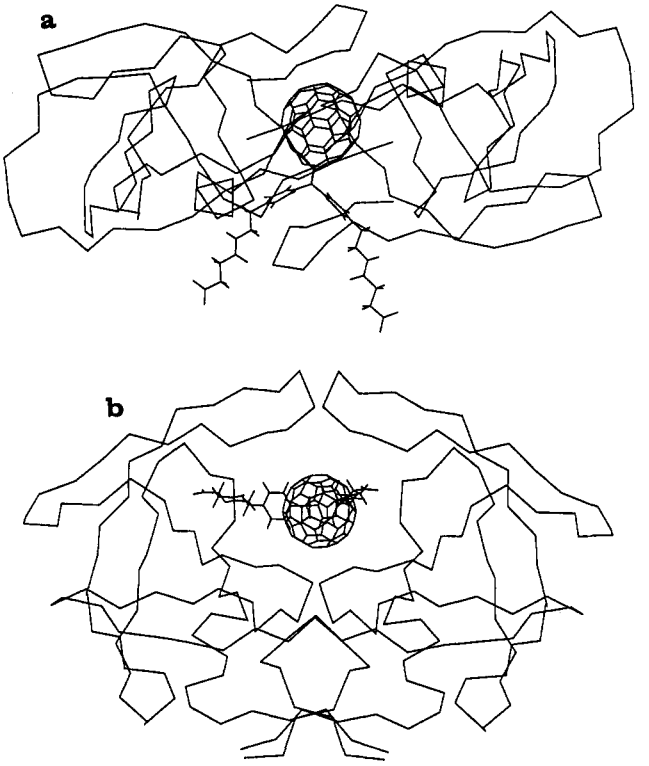


Figure 3. Top DOCK3 complex of compound 1 in (a) "front" and (b) "top" views. For clarity, only the α-carbon chain trace of HIVP is shown.

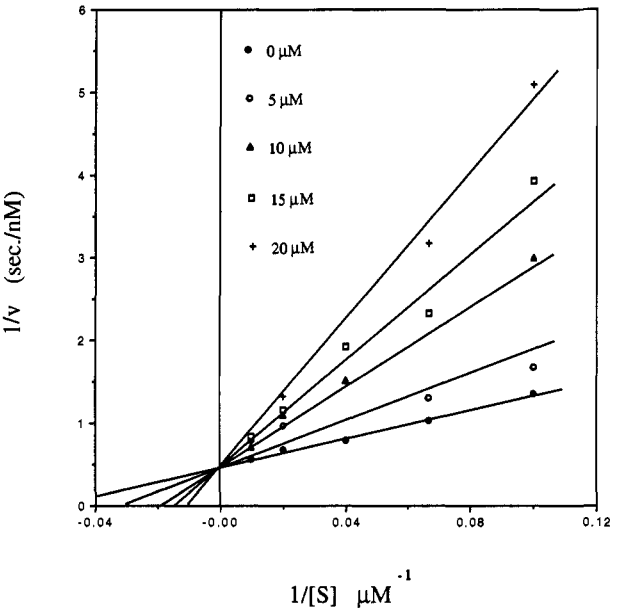


Figure 4. Double reciprocal plot of compound 1 inhibition of HIVP. Assays were performed in buffer containing 50 mM NaAc pH 5.5, 1.0 M NaCl, 5% glycerol, 1% DMSO, and 2 mM EDTA. Kinetic constants were determined by fitting of the data to the equation $v = V_mS/[K_m(1+I/K_i)+S]$ which describes competitive inhibition. K_i: 5.3 μM [0.98]. K_m: 15.9 μM [2.9]. V_m: 1.9 nM/s [0.1]. Standard errors are indicated in brackets.

that a possible mode of inhibition is through a tight electrostatic interaction of the dication with the catalytic aspartates. K_i values for these dications are in the micromolar range, corresponding to ~8 kcal/mol of binding energy, over and above the Gibbs energy loss due to freezing out translational entropy. If even a fraction of the binding energy due to this type of interaction can be added to the existing C_{60} core binding energy, improvements of several orders of magnitude should be realized. It has been shown that the introduction of a single amine/carboxylate salt bridge can increase the binding energy of a ligand to its receptor by ~4 kcal/mol,[14] leading to a 1000-fold improvement in binding.

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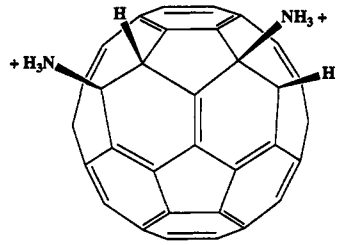

Figure 5. Compound **2**.

With these ideas in mind, we have investigated whether the active site of the HIVP can accommodate a C_{60} that has been derivatized with two amino groups and, in addition, if these groups can be positioned so that they are close enough to the catalytic aspartates to form effective salt bridges. Direct amino adducts can be prepared by reacting a neat amine with the C_{60}, although controlling the stoichiometry of this reaction poses practical problems.[15] Synthetic issues aside, we have modeled compound **2**, 1,4-diamino C_{60}, (Figure 5) and examined its possible interactions with the HIVP active site. DOCK3 is able to orient compound **2** within the active site, placing the core C_{60} in a similar position to that of compound **1**, again allowing extensive nonpolar van der Waals surface interaction. In addition, the two amino groups can effectively bridge the oxygens of the catalytic aspartates, approaching within 2.7 Å and 3.4 Å, respectively (N–O distance), thus making these amino/carboxyl interactions good candidates for improving overall binding (Figure 6).

Conclusions

We have suggested through modeling that C_{60} derivatives should be inhibitors of the HIVP due to their steric and chemical complementarity with the active site. We have demonstrated that this is the case and that this behavior is consistent with a qualitative analysis of hydrophobic surface transfer in model complexes.

Our interest in C_{60} derivatives is twofold. First of all, they represent nonpeptide-based lead compounds that, through careful modeling, may result in effective, tightly binding HIVP inhibitors. Second, they represent a rigid, conformationally restricted scaffold with which we can examine the nature of protein–ligand binding. Because of the steric bulk of C_{60} and its complementarity to the active site surface, there are severe limitations to the orientations it can adopt within the active site. Essentially, the principal degree of freedom of a C_{60} derivative within the active site is rotation around its center. This simplifies the problem of predicting the binding modes of various derivatives. The key to exploiting this system will be the development of the synthetic methodology to facilely and specifically modify the C_{60} surface.

Experimental Section

Modeling. All modeled compounds were generated using the SYBYL 5.4 package. Atomic point charges were calculated using the Gasteiger–Huckel method. For conformationally flexible ligands, torsions were initially set to anticipated low-energy conformers. Minimization to the used model structure was done using the Maximin2 minimizer and Tripos force field and parameters. Docking to the active site of the studied protein was done using the program DOCK3.[4] Grids required by DOCK3 were generated against the dimer formed from the Protein Data Bank file 3hvp, using the standard AMBER united atom charges and van der Waals parameters. Single mode runs of modeled compounds against the active site were performed using the following parameters: dislim = 1.500, nodlim = 5, ratiom = 0.0000, lownod = 4, lbinsz = 0.4000, lovlap = 0.1000, sbinsz = 0.8000, and sovlap = 0.2000. All molecular graphics were produced using the MIDAS Plus system. Molecular surfaces were generated using the program MS, written by Michael Connolly. A probe sphere diameter of 1.4 Å and default values for van der Waals radii were used.

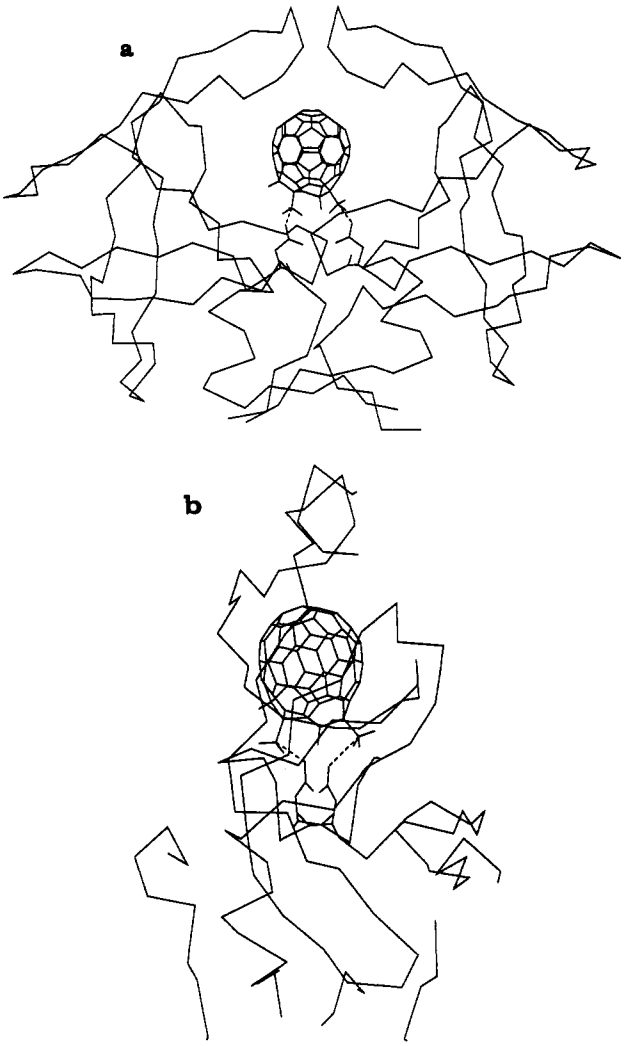

Figure 6. DOCK3 complex of compound **2** with the HIVP in (a) "front" and (b) "side" views. Close approach of compound **2** amino groups and HIVP catalytic aspartate oxygens is highlighted with dashed lines. For clarity, only the catalytic aspartates and the α-carbon chain trace of HIVP are shown.

Enzyme Assays. Compound **1** was assayed against recombinant, affinity-purified HIV-1 protease produced by Bachem Biosciences (0.16 mg protein/mL) at 25 °C. Assays were performed in 100 μL volumes under final conditions of 50 mM NaAc pH 5.5, 1.0 M NaCl, 5% glycerol, 1% DMSO, and 2 mM EDTA. Inhibitor was preincubated with ~0.05 μg of enzyme for 5 min, at which time the reaction was initiated by addition of substrate. The reaction was quenched at <15% product formation by the addition of 15 μL of 10% TFA. The cleavage products of the substrate peptide H-Lys-Ala-Arg-Val-Tyr-*p*-nitro-Phe-Glu-Ala-Ile-NH_2 (made by Bachem) were assayed by HPLC using a 10–40% (acetonitrile, 0.1% TFA):(water, 0.1% TFA) gradient over 30 min at 1 mL/min. Product was quantitated by integration of peak areas followed by comparison to product standard curves. Determination of kinetic constants was done with the program KinetAsyst (IntelliKinetics).

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Note Added in Proof: The parent compound to compound **1**, where X = $(CH_2)_2NH_2$, was tested and found to have a K_i of ~2 μM. This insensitivity of binding to the nature of the C_{60} side chain supports the predicted binding mode, which positions the side chains away from the active site into full solvent contact.

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