Screening Solution-Phase Combinatorial Libraries Using Pulsed Ultrafiltration/Electrospray Mass Spectrometry

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A method is described whereby a family of homologues is synthesized in a one-pot reaction, without isolation or purification, and the reaction mixture is screened using a competitive binding assay based on pulsed ultrafiltration/electrospray mass spectrometry (PUF/ESMS) to tentatively identify those derivatives having the highest affinity for a target receptor. As a model system to test this approach, a synthetic scheme designed to prepare a series of analogues of the adenosine deaminase inhibitor *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA), as diastereomeric mixtures, was carried out. Pulsed ultrafiltration screening of the crude reaction mixture against controls without protein detected protonated molecules corresponding to EHNA-type derivatives and three of its linear, alkyl homologues but did not show protonated molecules for an isobutyl or benzylic EHNA derivative, suggesting the latter was inactive. To verify this conclusion, we prepared E/THNA, the linear homologues, and the benzylic derivative (each as a diastereomeric mixture) and bioassayed them for their adenosine deaminase inhibition index ($[I]/[S]_{0.5}$). The bioassay results for the individually synthesized analogues were in good agreement with that predicted by the observed relative ion enhancement in the PUF experiments. Thus, the PUF protocol might be used as a general method to quickly provide direction to the chemist in search of drug candidates.

A most promising new approach to drug discovery concerns the synthesis and screening of combinatorial libraries in order to identify new compounds that express high affinity and specificity for a pharmacologically relevant, biomolecular target. Advances in molecular biology, automated chemical synthesis, and robotics have facilitated the formulation of vast libraries of structurally related molecules.[1]

An essential aspect of screening large combinatorial libraries is the ability to identify the active components in these complex mixtures, which is usually based on the strength of binding to a selected target macromolecule. A common approach to this problem has utilized immobilization of either the target receptor or library molecules on a solid-phase support in order to facilitate identification of the active compounds in the library.[2,3] Technically this is also true of the phage-related methodologies.[4] Another approach is the iterative resynthesis of components of the library with progressively lower complexity until a single most active compound is identified.[5,6] However, immobilization may change the affinity characteristics of the binding ligand or the receptor from its native, solution-phase form, and the resynthesis approach is still labor-intensive and time-consuming. A method which would both facilitate the screening of solution-phase libraries and target molecules with a minimum amount of sample handling and also permit a rank ordering of binding constants of target and library molecules in solution would be of value.

We are developing techniques with these goals in mind, using a combination of pulsed ultrafiltration (PUF) and electrospray mass spectrometry.[7] Pulsed ultrafiltration is a technique under development in our laboratory for the quantitative measurement of macromolecule/ligand interactions. During pulsed ultrafiltration, a macromolecular target is trapped in a flow-through cell by an ultrafiltration membrane; then an aliquot of a ligand is pumped through the ultrafiltration cell. As ligands bind and dissociate from the solution-phase receptors (macromolecules or complexes thereof), their elution profile from the ultrafiltration cell is altered relative to control. We have developed solutions to the differential equations describing this interaction which in individual experiments have allowed us to conveniently measure classical binding parameters, i.e., K_d, K_i (competitive binding), n (number of binding sites), ΔH, ΔS of binding, and in the case of enzymes, Michaelis−Menten constants, K_m, are projected.[8]

By coupling the pulsed ultrafiltration cell to an electrospray mass spectrometer detector, ligand/biomolecule interactions can be analyzed using extraordinarily small amounts of material while concurrently providing structural information on the ligand eluate. During these studies, we realized that this system might address the aforementioned problems associated with identification of ligands of biological interest in a combinatorial library and that this analysis could be carried out with both ligand and macromolecule free in solution. Thus, a combinatorial library pulse passed through an ultrafiltration cell containing the target macromolecule would show elution profile perturbation, as a function of affinity, of that eluate ligand which interacted with the macromolecule. In preliminary work we have shown that we can adjust this system to

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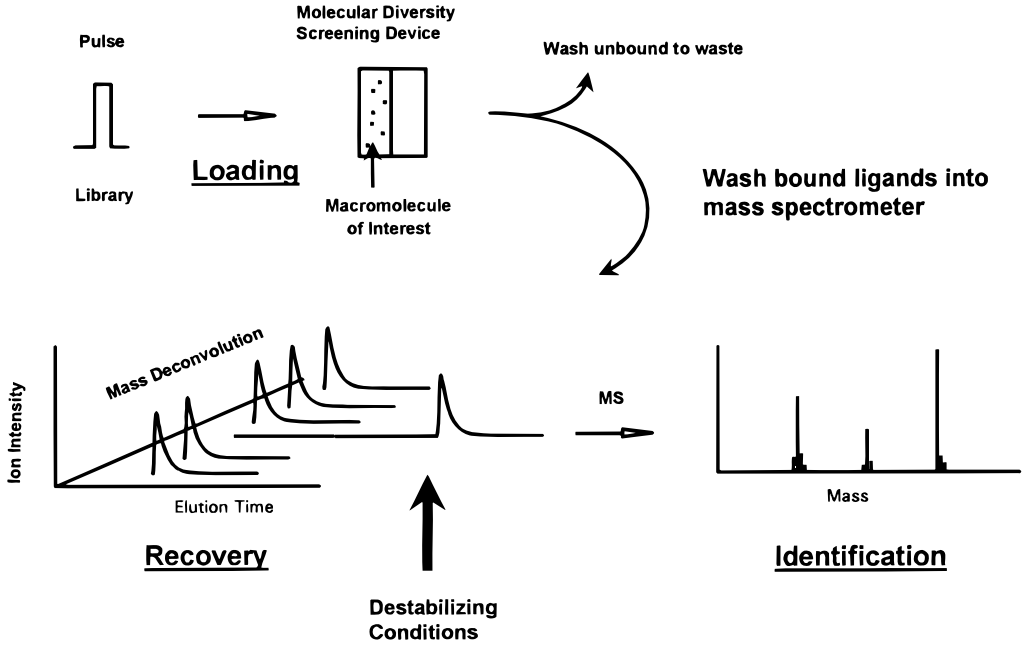


Figure 1. Schematic representation of the PUF molecular diversity screening method.

completely separate ligands which have high affinity and specificity for the target macromolecule compared to those which are nonbound or weakly bound, i.e., to identify the "needle in the haystack".[7] In the present work, we apply an experimental protocol where the ligand/receptor is first incubated off-line and washed and then the ligands are released into the mass spectrometer for identification.

Results and Discussion

The general protocol for this screening procedure is schematically outlined in Figure 1. In the present work, we investigated whether this system might be applied to the screening of solution-phase libraries produced by conventional synthetic means. The general idea was to apply a synthetic scheme which would likely produce a family of homologues in a one-pot reaction and then, without further characterization, screen the crude mixture using the PUF/MS system. Potentially interesting compounds identified by their mass would then be further characterized, thus focusing the characterization effort only on those compounds of interest.

A full understanding of the PUF approach to screening requires an intimate and quantitative understanding of many interrelated variables, e.g., ligand affinity constant, the efflux of unbound compound from the cell, cell mixing and flow rate, receptor concentration, individual library compound concentrations, protein polarization on the membrane, and ionization during electrospray. However, much can be gleaned by assuming the system is first brought to a steady-state condition, where the amount of bound ligand is defined by the law of mass action, and then allowing the system to be perturbed releasing bound ligands. Thus, if the binding cell in Figure 1 is filled with macromolecule of concentration $[M_0]$ and then eluted with a library ligand of concentration $[L_0]$ until steady state is reached, the effluent from the cell and the free ligand concentration in the cell will have a ligand concentration of $[L_0]$. Under these conditions, the concentration of ligand bound, $[L_b]$, will be governed by the law of mass action:

$$[L_b] = \frac{[L_0][M_0]K_a}{1 + [L_0]K_a}$$

where K_a is the association constant of the ligand for the macromolecule, when there is a single binding site for the ligand.

Extension of this analysis suggests that when the receptor is challenged with saturating amounts of ligands, the concentration of ligands in the release phase will be proportional to their affinities (for each class of compound competing for a single binding site). Besides identifying the ligands upon their release from the PUF chamber, electrospray mass spectrometry may be used to roughly estimate the relative concentrations and therefore affinities of each ligand by comparing the relative increase in ion abundances in the release phase to those in the original library mixture. Within certain limitations, one might use the PUF approach to quickly assess which compounds in a mixture have the highest affinity for a receptor, even in a crude reaction mixture. Quantitative conclusions about ligand macromolecule interactions assume a completely homogeneous mixture of ligand and macromolecule during the PUF analysis. Consequently in the present case, where the binding cell is unstirred, this is only likely to be true in a relative sense.

As a model system we chose adenosine deaminase (ADA) and derivatives of the inhibitor EHNA [(+)-*erythro*-9-(2-hydroxy-3-nonyl)adenine, $K_d = 7 \times 10^{-9}$ M].[9] ADA is an enzyme which metabolizes adenosine and is of some significance in the inactivation of adenosine analogues used in cancer and AIDS treatment. A number of analogues have been developed for the inhibition of this enzyme, including EHNA.

Synthesis of potential EHNA derivatives was carried out in three steps as shown in Figure 2. An equimolar mixture of the eight methyl ketones was brominated

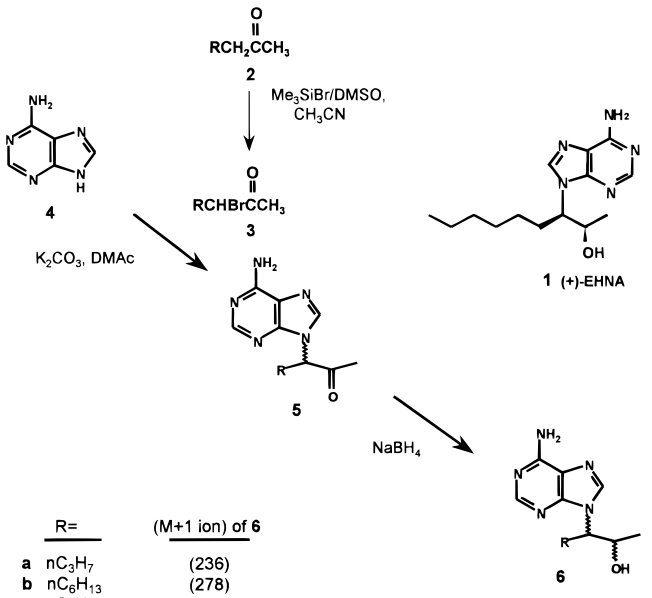


Figure 2. Synthesis of EHNA analogues. For the same "pot" reaction, all eight side chains were introduced into the syntheses at the same time and were themselves prepared in one "pot". Isolations of individual compounds were not performed. The only purification was simple liquid−liquid extraction. Derivatives **6b**,**d**,**h** were also synthesized individually (as their diastereomers) in the conventional manner and tested for ADA inhibition (Table 2).

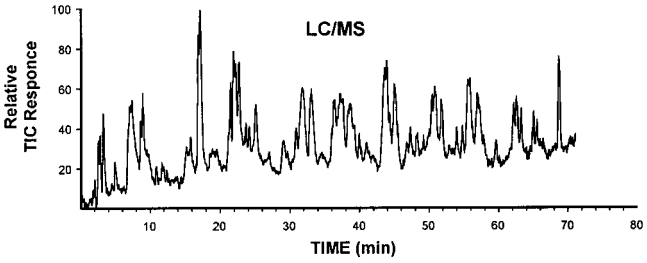


Figure 3. Reverse-phase LC−MS profile of crude EHNA library as prepared in Figure 2.

with trimethylbromosilane−dimethyl sulfoxide in dry acetonitrile at room temperature. Adenine was then treated with 2 mol equiv of the crude α-bromo ketones (assumed) in *N*,*N*-dimethylacetamide in the presence of potassium carbonate, and the presumed intermediate ketones were reduced using sodium borohydride.

All three reactions were carried out without any attempt to isolate intermediates or final products, other than to remove salts and solvent. In addition to forming multiple products by virtue of multiple competing reagents in the same reaction vessel, the chemistry outlined could produce many byproducts due to side reactions. Thus, for each R group, four isomers are expected about the two chiral centers in the molecule. Further, other products might be expected by virtue of side reactions from alkylation of other nitrogens in the molecule and by other unidentified side reactions. As shown in Figure 3, a positive ion electrospray LC−MS analysis of this crude reaction mixture showed considerable chemical heterogeneity. Nevertheless, computer-reconstructed mass chromatograms (Figure 4) indicated that protonated molecules consistent with seven of the potential products were present at approximately equal abundance (each of the doublets probably represents a set of diastereomers, data not shown for $-nC_3H_7$ product). A mass corresponding to the eighth product, $R = (CH_3)_2C{=}CH{-}CH_2{-}$, did not appear in the crude reaction mixture.

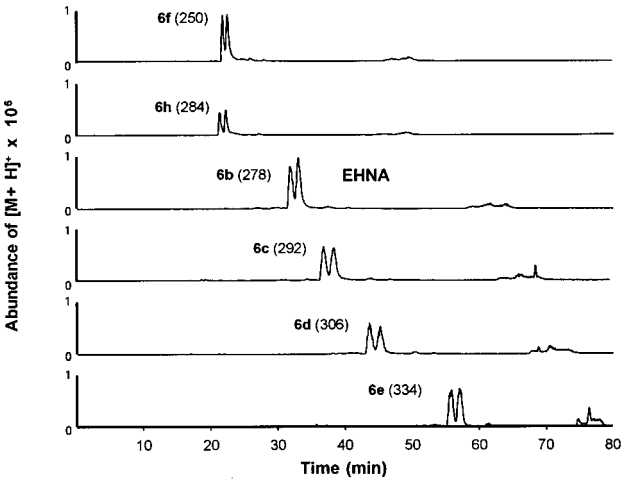
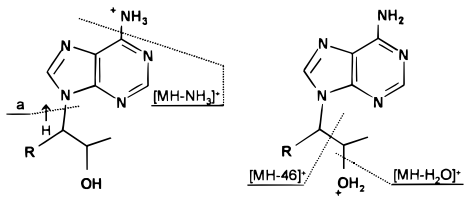


Figure 4. Computer-reconstructed mass chromatograms (from LC−MS analysis in Figure 3) showing $[M + H]^+$ ions of EHNA type analogues expected from the reaction given in Figure 2. Each doublet represents four stereoisomers.

Table 1. Electrospray Tandem Mass Spectrometry of Synthetic EHNA Library[a]



library compd (mass)	ion fragment (*m*/*z*)			
	a	$[MH - 46]^+$	$[MH - H_2O]^+$	$[MH - NH_3]^+$
6b (278)	136 (100)	232 (15.3)	260 (11.4)	261 (8.2)
6h (284)	136 (100)	238 (6.2)	266 (29.8)	267 (24.8)
6c (292)	136 (100)	246 (2.0)	274 (5.4)	275 (1.3)
6d (306)	136 (100)	260 (1.8)	288 (5.7)	289 (1.3)
6e (334)	136 (100)	288 (4.0)	316 (6.6)	317 (2.0)

[a] Library was analyzed as a mixture without chromatographic separation. Masses corresponding to all expected library compounds were observed in the positive ion electrospray mass spectrum of the mixture except the compound at *m*/*z* 250 (see LC−MS analysis in Figure 5).

In addition to molecular weight confirmation of seven EHNA analogues in the reaction mixture using LC−MS, electrospray tandem mass spectra were obtained in another set of analyses which provided additional structural information of components of the library. A summary of fragment ions common to all the EHNA analogues is shown in Table 1. Fragment ions were formed following protonation at either the primary amino group or the hydroxyl group as indicated in the structures in Table 1. The most abundant fragment ion in each tandem mass spectrum corresponded to protonated adenine, *m*/*z* 136, formed by elimination of the substituent group from the adenine nitrogen (see structure in Table 1). Other significant fragment ions

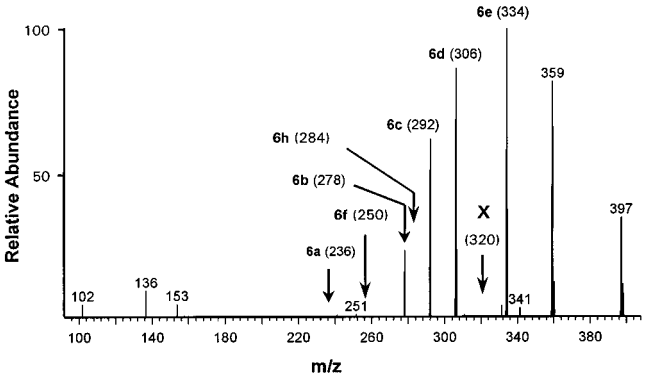


Figure 5. Protonated molecules released from ADA enzyme and detected using the PUF/ES MS protocol outlined in Figure 1 (scan mode). Ions at *m/z* 359 and 397 are not present in significant concentration in the library and are considered artifacts.

included $[MH - H_2O]^+$ and $[MH - 46]^+$, which confirmed the presence of a hydroxyl group on the acyclic side chain of adenine and loss of ammonia from the protonated molecule, consistent with the presence of a primary amine.

An advantage to this approach for screening is that full structure elucidation of analogues is not carried out until potential targets are identified. PUF/MS takes advantage of the specificity of mass spectrometry and the selectivity of PUF to quickly identify potential lead compounds in crude reaction mixtures. Once a preliminary correlation of structure−activity has been established, full characterization need only be carried out on the smaller number of compounds of potential biological interest.

When ADA (approximately 1 nmol) was challenged with this crude reaction mixture using the general PUF screening protocol outlined in Figure 1, only certain compounds were released by aqueous acetonitrile after the initial wash phase (Figure 5), as identified by mass spectrometry. Thus, $[M + H]^+$ ions corresponding to protonated EHNA (*m/z* 278) and three of its putative homologues (nC_7H_{15}, *m/z* 292; nC_8H_{17}, *m/z* 306; and $nC_{10}H_{21}$, *m/z* 334; see Figure 2 for structures) were detected eluting from the cell in the recovery phase. However, protonated ions corresponding to the *n*-propyl (*m/z* 236), isobutyl (*m/z* 250), and benzylic (*m/z* 284) derivatives were not detected in these scans when compared against appropriate controls, even though their presence was observed in the reaction mixture before addition of protein. There is little or no signal at *m/z* 320 corresponding to the nC_9H_{19} homologue (Figure 5). This particular side chain was not introduced as a reactant and thus acts as a negative control for the experiment. The ions at *m/z* 359 and 397 are considered to be artifacts, since they are not present in significant concentration in the library control experiments and were known to be significant background ions in this instrument.

A similar series of experiments was carried out using positive ion electrospray mass spectrometry, with selected ion monitoring. In these experiments, protonated molecules corresponding to four of the seven EHNA-type analogues in the reaction mixture were monitored in the presence (Figure 6b) and absence (Figure 6a) of ADA and finally with a 6-fold excess of authentic (±)-EHNA added (relative to protein, Figure 6c). Results similar

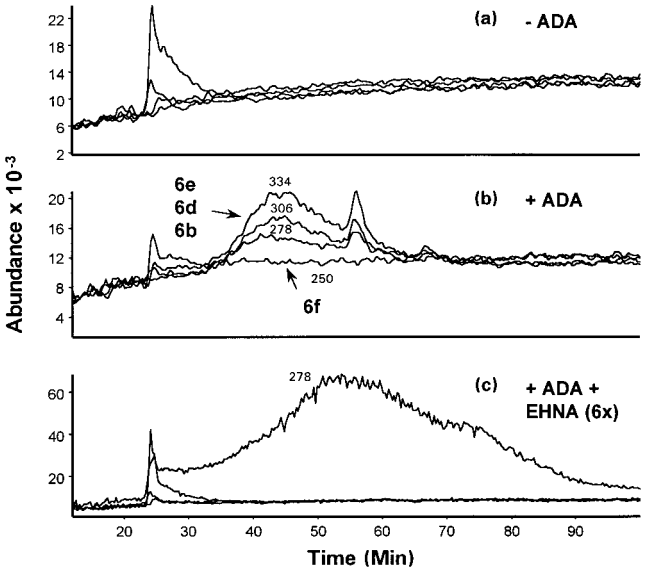


Figure 6. Selected ion chromatograms (*m/z* 250, 278, 306, and 334) of protonated library molecules washed from the binding cell with methanol using the PUF/ES MS protocol outlined in Figure 1: (a) in the absence of ADA, (b) in the presence of ADA, and (c) in the presence of ADA with a 6-fold excess of EHNA. The methanol wash (destabilizing condition) starts at time = 0.

Table 2. Comparison of Observed PUF Binding Enhancement of EHNA Analogues by ADA with the Biological Activity of Selected Diastereomeric Pairs Synthesized Individually

diastereomeric compd (M + 1) ion	ADA inhibtn index ($[I]/[S]_{0.5}$)	inhibtn of ADA relative to E/THNA = 1	relative ion enhancement by PUF[a]
6b (278)	0.00042	1.0	1.0
6d (306)	0.00016	2.6	5.3
6h (284)	0.069	0.006	<0.01

[a] Relative ion enhancement caused by the PUF process was determined by measuring the increase in signal abundance relative to that corresponding to E/THNA in Figure 5 (representing PUF enhancement of bound derivatives) versus the relative abundance of the same signal to E/THNA derivatives in Figure 4 (relative concentration of derivatives in reaction mixture before PUF).

to the above were obtained for ions in the presence of ADA versus control, where only (±)-EHNA and its linear homologues were observed to bind. Further, in the presence of the excess EHNA, signals corresponding to the protonated ions of the linear homologues (R = nC_8H_{17}− and R = $C_{10}H_{21}$−) were completely suppressed. This suggests that binding of these compounds was competitive with EHNA for the enzyme. The initial burst of compounds at about 24 min is thought to be due to nonspecific binding of the EHNA compounds to the membrane and cell parts.

Taken at face value, these PUF experiments can provide a first-approximation structure−activity relationship (SAR) analysis of synthetic analogues produced in the one-pot reaction. In general, the greater the enhancement of a particular derivative in the eluate from the binding cell in the release phase relative to its starting concentration in the library, the greater its expected interaction with the receptor protein in the cell (see previous discussion on stirring and competitive interaction at a single site).

Table 2 gives the relative enhancement of the ion currents corresponding to the higher alkyl homologue (**6d**) and benzylic derivative (**6h**) versus the E/THNA diastereomers (**6b**) with the latter enhancement being

assigned a value of 1. As may be seen, the *m/z* 306 ion (alkyl homologue **6d**) is enhanced by a factor of about 5, whereas the *m/z* 284 ion (benzylic derivative **6h**) shows less than 1/100 the enhancement of the E/THNA derivatives. Given the fact that these compounds appear to be acting at the active site on the enzyme (see previous discussion of competition with authentic EHNA), this would strongly suggest that the benzylic derivative (**6h**) is inactive relative to the EHNA family and as a first approximation that the higher homologues may be more active.

To verify these conclusions, we prepared the diastereomers of EHNA (i.e., EHNA and THNA, **6b**), the linear homologues **6d**, and the benzylic derivative **6h** and bioassayed them for their ADA inhibition index ($[I]/[S]_{0.5}$) using the method of Schaeffer.[10] The synthetic approach was the same as for the one-pot reaction except that the reactions were carried out individually and the intermediates were purified by chromatography. The final derivatives (**6b**,**d**,**h**) were greater than 99% pure by HPLC (UV detection) and showed the expected ESMS signals at *m/z* 278, 306, and 284, respectively, as the only ion currents. As may be seen in Table 2, relative to an E/THNA activity of 1, the derivative **6d** is more than 2 times as active and the benzylic derivative **6h** has about 6/1000 the activity of the EHNA family. These results are in good agreement with that predicted by the observed relative ion enhancement in the PUF experiments, also shown in Table 2.

The synthesis and PUF screening of the EHNA derivatives presented in this paper can be carried out by one person in approximately 2−3 days with about equal time spent on the chemistry and mass spectrometry. To the extent that such a system is generally applicable, standard solution-phase chemistry might effectively be used in combination with PUF/electrospray mass spectrometry to quickly provide a first-line screen of related derivatives and direction to the chemist in search of biologically active compounds.

Experimental Section

(±)-EHNA was purchased from Sigma Chemical Co. (St. Louis, MO). Adenosine deaminase enzyme (ADA) was purchased from Boehringer Mannheim (Indianapolis, IN) as a suspension in 3.2 M $(NH_4)_2SO_4$. The ADA was pelleted by centrifugation, the supernatant decanted, and the pellet dissolved in phosphate buffer before being used. Enzyme concentrations were measured by the BCA method using a commercially available kit from Pierce (Rockford, IL). Molarity of the enzyme was calculated using a MW of 41 250.5, which was determined by using matrix-assisted laser desorption time-of-flight mass spectrometry.[7]

Reagents used in the synthesis were purchased from Aldrich Chemical Co. (Milwaukee, WI). Column chromatography was monitored by TLC (silica gel 60 F_{254}; Rieldel-de Haën AG, Germany). Silica gel for column chromatography was purchased from Sigma Chemical Co. (St. Louis, MO; 10−40 μm for flash chromatography and 200−400 mesh for open column). TLC plates were visualized with UV light or by treatment with phosphomolybdic acid (PMA). All single intermediates and final products were judged homogeneous by TLC and characterized by NMR and MS. Individually synthesized final diastereomers were also subjected to HPLC, using a Hypersil BDS-C_{18} 2- × 250-mm column with 5-μm particle (Hewlett-Packard) at a flow rate of 0.22 mL/min. For the library, a Hypersil C_{18} 4- × 100-mm column (Hewlett-Packard) was used and LC carried out at 0.60 mL/min. HPLC grade solvents were obtained from Fisher Scientific Inc. (Itasca, IL). NMR spectra were recorded on a Varian XL 300 spectrometer (300 MHz). Chemical shifts are denoted in ppm (δ) relative to TMS as the internal reference. Unless otherwise noted the NMR spectra were obtained using $CDCl_3$ as the solvent.

Synthetic mass spectra were obtained on either a Finnigan MAT 90 double-focusing mass spectrometer or the Hewlett-Packard mass spectrometer described below. Tandem MS data were acquired using a Bruker (Billerica Mall, MA) ESQUIRE ion trap mass spectrometer equipped with electrospray ionization.

The pumping and injection apparatus for the mass spectrometer interface consisted of a Hewlett-Packard (Palo Alto, CA) 1090L gradient HPLC system (50 μL/min) equipped with a Rheodyne (Cotati, CA) model 8125 injector and photodiode array UV/vis absorbance detector. UV data were collected by monitoring absorbance at 260 nm. Mass spectrometry was carried out with a Hewlett-Packard mass spectrometer equipped with a ChemStation data system and nebulizer-assisted electrospray LC−MS interface. During analysis the quadrupole analyzer was maintained at 120 °C. Nitrogen (80 psi) was used for nebulization of the eluate, and the nitrogen bath gas, for evaporation of solvent from the electrospray, was held at 300 °C and a flow rate of 10 L/min.

Binding Cell. The binding cell was fashioned from an HPLC preparative in-line solvent filter (Upchurch Scientific, Oak Harbor, WA; cat. #A-333) in which the filter element was replaced by a semipermeable membrane (YM10, MWCO 10 000; Amicon, Beverly, MA), which was cut to exactly the same size as the filter element. When the membrane was inserted between the two internal halves of the cell body and the cell assembled with the outer compression elements, the system formed two internal chambers of different sizes, again separated by the semipermeable membrane. The larger of the two chambers was used as the inlet side (where the enzyme was held) and the smaller chamber the outlet side of the binding cell. Both the inlet and outlet were connected to 0.005-in. i.d. tubing (PEEK, Upchurch) via finger tight fittings (Upchurch). To enhance flow from the outlet side of the cell, small (ca. 0.001 in.) grooves were cut in the outlet face of the cell body. These grooves were radial in nature, terminating at the center hole for the outlet port. The total volume of the larger chamber in the cell in this configuration was approximately 80 μL.

One-Pot Synthesis of EHNA Analogues. The α-halo ketones were prepared as a mixture using the trimethylbromosilane−dimethyl sulfoxide method of Pagnoni.[11] Briefly, to a stirred solution of a mixture of eight methyl ketones (1 mmol each) in dry acetonitrile (20 mL) were added trimethylbromosilane (1.16 mL, 8.8 mmol) and then dimethyl sulfoxide (0.63 mL, 8.8 mmol) dropwise. The eight ketones consisted of 2-hexanone, 2-nonanone, 2-decanone, 2-undecanone, 2-tridecanone, 5-methyl-2-hexanone, 6-methyl-5-hepten-2-one, and benzylacetone. The reaction mixture was kept at room temperature for 2 h, poured into water (80 mL), and subsequently extracted with ethyl ether (3 × 35 mL). The ether layer was dried over anhydrous sodium sulfate and then evaporated in vacuo to a brown liquid which was used without further purification.

A mixture of the α-bromo ketones (assumed 8 mmol), adenine (540 mg, 4 mmol), anhydrous potassium carbonate (552 mg, 4 mmol), and *N*,*N*-dimethylacetamide (20 mL) were added together and then stirred at 110 °C for 1 h. After cooling, the reaction mixture was filtered, and the filtrate was evaporated in vacuo to dryness giving a brown solid which was used without further purification in the following reduction step.

A mixture of $NaBH_4$ (610 mg, 16 mmol) and the brown solid obtained from the previous alkylation step (assumed 8 mmol) in ethanol (25 mL) was allowed to stir at room temperature for 1 h. The reaction was then quenched with acetic acid and neutralized with saturated $NaHCO_3$ and the majority of the ethanol removed in vacuo. After dilution with water, the mixture was extracted with ethyl ether (3 × 10 mL). The ether layer was dried and evaporated in vacuo to produce a yellow liquid which was used directly in the binding studies.

This yellow liquid was analyzed by reverse-phase electrospray LC−MS. Thus, 3 μL of the oil diluted 20× with

methanol was injected onto a C_{18} column (Hewlett-Packard Hypersil, 10 cm × 4 mm) attached to the aforementioned electrospray mass spectrometer system. The column was eluted with a water(A)/methanol(B) linear gradient (12−45% B, 0−20 min; 45−85% B, 20−70 min; 85−95% B, 70−75 min; and 95% B, isocratic) at a flow rate of 60 μL/min. Mass spectra were scanned from 100 to 500 amu at a rate of 1 scan/s. The results of these studies are shown in Figure 3. The MS/MS analyses were carried out by direct infusion of the yellow liquid in a solution of acetonitrile/water (1:1, v/v) containing 1% acetic acid at 10 μL/min. The results of this study are summarized in Table 1.

Synthesis of Selected Diastereomeric EHNA Analogues. 9-(2-Hydroxy-3-nonyl)adenine (6b). The required α-halo reagent **3b** was prepared by the trimethylbromosilane−dimethyl sulfoxide method of Pagnoni.[12] Briefly, trimethylbromosilane (1.0 mL, 7.58 mmol) and then DMSO (0.54, 7.58 mmol) were added slowly at room temperature to a stirred solution of 2-nonanone (**2b**; 0.98 g, 6.89 mmol) in dry acetonitrile (20 mL). The mixture was stirred for 2 h, poured into ice-cold water (50 mL), and extracted with $CHCl_3$ (3 × 20 mL). The organic layer was dried (Na_2SO_4) and evaporated to give a residue which was subjected to column chromatography (hexane/$CHCl_3$, 3:2) to yield 3-bromo-2-nonanone (**3b**; 0.76 g, 50%): TLC R_f = 0.44 ($CHCl_3$/hexane, 1:1); NMR δ_H 0.89 (3H, t, CH_3), 1.30 (12H, m, 4 CH_2), 1.96 (2H, m, CH_2), 2.37 (3H, s, CH_3), 4.23 (1H, t, CH).

A stirred mixture of the 3-bromo-2-nonanone (**3b**; 448 mg, 2 mmol), anhydrous K_2CO_3 (248 mg, 1.8 mmol), and adenine (**4**; 243 mg, 1.8 mmol) in *N*,*N*-dimethylacetamide (5 mL) was kept at 70 °C for 30 min. The insoluble solids were then filtered and washed with hot ethanol. Ethanol from the filtrate and *N*,*N*-dimethylacetamide were removed under vacuum, and the brown residue so obtained was purified by flash chromatography (EtOAc/MeOH, 93:7) to give 9-(2-keto-3-nonyl)adenine (**5b**; 274 mg, 55%): TLC R_f = 0.55 (EtOAc/MeOH, 93:7); NMR δ_H 0.84 (3H, t, CH_3), 1.22 (8H, m, 4CH_2), 2.05, 2.27 (2H, m, CH_2), 2.28 (3H, s, CH_3), 5.39 (1H, q, CH), 6.76 (2H, s, NH_2), 7.98 (1H, s, Ade-2 or -8H), 8.35 (1H, s, Ade-2 or -8H); δ_C ($CDCl_3$) 13.93, 22.39, 25.83, 27.64, 28.52, 30.99, 31.33 ($CH_3(CH_2)_5$, CH_3), 62.34 ($C_6H_{13}CH$(Ade)CO-), 119.05, 139.40, 150.06, 153.01, 155.98 (five Ade ring carbons), 203.46 (CH_3CO).

To a solution of keto derivative **5b** (186 mg, 0.68 mmol) in EtOH (3 mL) was added aqueous $NaBH_4$ (3 mL, 9 mg, 0.24 mmol). The mixture was stirred at room temperature for 1.5 h and then the reaction quenched by adding acetic acid. The solution was neutralized with saturated aqueous $NaHCO_3$ and then extracted with ethyl acetate (3 × 15 mL). The organic phase was dried ($MgSO_4$) and then evaporated to give a diasteromeric mixture of 9-(2-hydroxy-3-nonyl)adenine (**6b**; 187 mg, 100%): TLC one spot, R_f = 0.41 (ethyl acetate/ethanol, 9:1); HPLC >99.0% (42% MeOH/H_2O, 12.1 and 13.1 min, corresponding to the diastereomers); ESMS gave a single ion current at *m*/*z* 278; NMR δ_H 0.78 (3H, t, CH_3), 1.08/1.28, 1.18 (11H, 2d, 1m, C*H*$_3$CH(OH), 4C*H*$_2$), 1.96, 2.06 (2H, m, C*H*$_2$CH(Ade)), 4.25 (1H, m, CH_3CH(OH) or $C_6H_{13}CH$(Ade)), 4.40 (1H, m, CH_3CH(OH) or $C_6H_{13}CH$(Ade)), 5.3 (1H, broad, OH), 6.92 (2H, s, NH_2), 7.90/7.95 (1H, 2s, Ade-2 or -8H), 8.28 (1H, s, Ade-2 or -8H); δ_C 13.83, 19.96/20.65, 22.32, 25.98, 27.37, 28.67, 31.37/31.58 ($CH_3(CH_2)_5$, CH_3), 61.93/62.26, 68.23/69.09 (CH(OH)CH(Ade)C_6H_{13}), 118.93/119.19, 140.00/140.52, 149.53/149.83, 152.20, 155.75/155.79 (five Ade ring carbons); HRMS (EI) for $C_{14}H_{23}N_5O$ calcd 277.1903, found 277.1884.

9-(2-Hydroxy-3-undecanyl)adenine (6d). The diastereomeric mixture of EHNA-type analogues **6d** and its intermediates were prepared in a manner similar to the nonane derivatives described above. Bromination of 2-undecanone (**2d**; 1.03 mL, 5 mmol) in dry acetonitrile (10 mL) gave 3-bromo-2-undecanone (**3d**; 0.80 g, 65%) as a colorless oil after chromatography (cyclohexane/$CHCl_3$, 3:2): TLC (cyclohexane/$CHCl_3$, 3:2) R_f = 0.50; NMR δ_H 0.88 (3H, t, CH_3), 1.27 (12H, m, 6CH_2), 1.96 (2H, m, CH_2), 2.36 (3H, s, CH_3), 4.22 (1H, t, CH).[12]

Alkylation of adenine (**4**; 71 mg, 0.53 mmol) with 3-bromo-2-undecanone (**3d**; 174 mg, 0.70 mmol) gave 9-(2-keto-3-undecanyl)adenine (**5d**; 90 mg, 56%) as a white solid after purification by flash chromatography: TLC ($CHCl_3$/MeOH, 9:1) R_f = 0.52; NMR δ_H 0.85 (3H, t, CH_3), 1.21 (12H, m, 6CH_2), 2.05, 2.26 (2H, m, CH_2), 2.28 (3H, s, CH_3), 5.38 (1H, q, CH), 6.56 (2H, s, NH_2), 7.97 (1H, s, Ade-2 or -8H), 8.35 (1H, s, Ade-2 or -8H); δ_C 13.99, 22.50, 25.80, 27.59, 28.79, 28.97, 29.06, 30.94, 31.61 ($CH_3(CH_2)_7$, CH_3), 62.27 (-*C*H(Ade)CO-), 118.97, 139.38, 150.01, 152.92, 155.77 (Ade 5 C's), 203.34 (-$COCH_3$).

Reduction of **5d** (89 mg, 0.29 mmol) in EtOH (3 mL) with $NaBH_4$ (4 mg, 0.11 mmol) gave the diastereomeric mixture of 9-(2-hydroxy-3-undecanyl)adenines as a white solid (**6d**; 89 mg, 100%): TLC one spot, R_f = 0.41 (ethyl acetate/ethanol, 9:1); HPLC >99.0% (75% MeOH/H_2O, 20.4 and 22.9 min, corresponding to the diastereomers); ESMS gave a single ion current at *m*/*z* 306; NMR δ_H 0.85 (3H, t, CH_3), 1.08/1.29, 1.19 (15H, 2d, 1m, C*H*$_3$CH(OH), 6C*H*$_2$), 1.98, 2.08 (2H, m, C*H*$_2$CH(Ade)), 4.26 (1H, m, CH_3CH(OH) or $C_8H_{17}CH$(Ade)), 4.38 (1H, m, CH_3CH(OH) or $C_8H_{17}CH$(Ade)), 6.70 (2H, s, NH_2), 7.86/7.91 (1H, 2s, Ade-2 or -8H), 8.28 (1H, s, Ade-2 or -8H); δ_C 13.99, 20.10, 20.77, 22.51, 26.12, 27.29, 29.04/29.08, 29.20/29.22, 31.66 ($CH_3(CH_2)_7$, CH_3), 62.31/62.71, 68.37/69.26 (CH(OH)CH(Ade)C_8H_{17}), 119.22/119.48, 140.19/140.69, 149.58/149.84, 152.30/152.35, 155.79/155.82 (five Ade ring carbons); HRMS (EI) for $C_{16}H_{27}N_5O$ calcd 305.2216, found 305.2208.

9-(2-Hydroxy-3-benzyl)adenine (6h). The diastereomeric mixture of aromatic EHNA analogues **6h** and its intermediates were prepared in a manner similar to the undecanyl analogues **6d**, described above. Bromination of the ketone (1.58 g, 10.7 mmol) gave, after column chromatography (hexane/EtOAc, 10:1), 3-bromo-benzylacetone (**3h**; 1.38 g, 6.1 mmol, 67%) as a colorless oil: TLC single spot, R_f = 0.49 (hexanes/EtOAc, 10:1); NMR δ_H 2.32 (3H, s, CH_3CO), 3.31 (2H, dd, $PhCH_2$), 4.47 (1H, t, CHBr), 7.19−7.35 (5H, m, C_6H_5).

Alkylation of adenine (718 mg, 5.32 mmol) with the bromoketone **3h** (158 mg, 0.70 mmol) yielded 9-(2-keto-3-benzyl)-adenine (**5h**; 793 mg, 53%) after column chromatography (EtOAc/EtOH, 9:1): TLC one spot, R_f = 0.36 (EtOAc/EtOH, 9:1); NMR δ_H 2.21 (3H, s, CH_3CO), 3.47 (2H, dd, $PhCH_2$), 5.51 (1H, q, CH(Ade)CO), 6.10 (2H, s, NH_2), 7.01−7.22 (5H, m, C_6H_5), 7.76 (1H, s, Ade-2 or -8H), 8.32 (1H, s, Ade-2 or -8H); δ_C 28.25 (CH_3), 36.91 (CH_2), 63.74/63.90 (-*C*HCOCH_3), 127.38, 128.71, 128.84, 135.25 (benzene 6 C's), 119.20, 139.67, 150.00, 153.01, 155.57 (Ade 5 C's), 202.93 (-*C*OCH_3).

Reduction of **5h** (320 mg, 1.14 mmol) with $NaBH_4$ (15.6 mg, 0.41 mmol) gave 9-(2-hydroxy-3-benzyl)adenine (**6h**; 290 mg, 90%): TLC R_f = 0.27 (EtOAc/EtOH, 9:1); HPLC purity >99.0% (30% MeOH/H_2O, 12.6 and 13.8 min, two diastereomers); ESMS gave a single ion current at *m*/*z* 284; NMR δ_H 1.07/1.43 (3H, 2d, CH_3), 3.30 (2H, t, CH_2), 4.25−4.52 (2H, m, BzC*H*(Ade)-C*H*(OH)CH_3), 6.30/6.36 (2H, 2s, NH_2), 6.80−6.84, 6.94−6.98, 7.14−7.19 (5H, m, benzene H), 7.50 (1H, s, Ade-2 or -8H), 8.31/8.32 (1H, 2s, Ade-2 or -8H); δ_c 20.59/20.92 (CH_3), 33.84/38.10 (CH_2), 65.16/66.54/67.80/68.84 (-*C*H(Ade)*C*H(OH)CH_3), 126.82/126.89, 128.66, 128.74, 137.24/137.45 (phenyl C), 119.56/119.85, 140.98/141.39, 149.00/149.23, 151.95/151.97, 155.63/155.69 (Ade C's); ESMS gave a single ion current at *m*/*z* 284 (M + 1); HRMS (EI) for $C_{15}H_{17}N_5O$ calcd 283.1433, found 283.1421.

Screening Protocol. Immediately before each experiment, the membrane was removed from its protective envelope and rinsed by floating glossy side down in a beaker of distilled water for 1 h while changing the water three times. After mounting in the ultrafiltration cell (glossy side toward the larger of the two chambers which will contain the enzyme), the membrane was flushed with 90% aqueous methanol or 90% aqueous acetonitrile for 15−20 min at 0.1 mL/min to remove organics present on its surface. The system was then equilibrated with binding buffer until UV detection showed a stable baseline.

The crude library oil as prepared above (100 μL) was dissolved in methanol (1 mL) and then diluted approximately 300-fold with potassium phosphate buffer (50 mM, pH 7.5). The control experiment was carried out first and was exactly the same as for the binding experiment except that buffer without enzyme was used. For the binding experiment, the aqueous buffer solution of the library (100 μL) was mixed with

phosphate buffer (150 μL) containing ADA enzyme (8.5 μM, total 1.02 nmol) and the mixture allowed to incubate at room temperature for 10 min before injection (200 μL) into the binding cell. The binding cell was then flushed with water at 100 μL/min to reduce salts and unbound and weakly bound ligands present in the library. To prevent buffer from entering the mass spectrometer during this wash phase, the flow was directed to waste. After 6 min of washing, the flow was directed to the mass spectrometer and the cell flushed for another 10 min with water. Mass spectra were collected by scanning the range m/z 100−400 over ca. 6 s at unit mass resolution. At 16-min postinjection, the eluant was changed to 90% acetonitrile at 50 μL/min (methanol produced similar results) to disrupt the ligand/enzyme complex and release ligands into the mass spectrometer as a bolus. To enhance electrospray ionization, water/acetonitrile/acetic acid (47.5:47.5:5, v/v/v) was added after the cell at 10 μL/min.

Compounds were analyzed using selected ion chromatograms to define their release after the changeover to the organic acetonitrile phase. Typically, there was a pronounced release of compounds when the acetonitrile first reached the binding cell. This initial signal then returned to baseline before the bound ligands began to elute at about 10 min after acetonitrile reached the binding chamber. This latter signal lasted about 25 min under the conditions used. Spectra were summed over this last area, and control spectra (summed over exactly the same time period) were subtracted. The resulting mass spectrum was reproducible and is shown in Figure 5.

Competitive Binding Protocol. In the competitive binding studies, three experiments were performed in which the library was subjected to the protocol described above: (1) without ADA enzyme, (2) with ADA enzyme, and (3) with ADA enzyme and an excess of externally added, authentic (±)-EHNA. Controls and data analyses were performed essentially the same as described above, except that (1) selected ion monitoring (2000-ms dwell time) was used for ion detection and (2) the library incubation was carried out in the presence of (±)-EHNA (4.15 nmol) equal to 6 times the enzyme concentration. The amount of enzyme used in these experiments was 0.69 nmol, and 90% methanol was used for the release phase. The results of these three experiments are presented in Figure 6.

Bioassay of Selected Diastereomeric Mixtures of EHNA Analogues. The inhibitory activity of three selected EHNA analogues, i.e., **6b**,**d**,**h**, was measured by the method of Schaeffer.[10] The inhibition index $[I]/[S]_{0.5}$, i.e., the ratio of concentration of inhibitor to that of substrate for 50% inhibition, was used to compare the inhibitory potency of the compounds. All compounds were assayed as a mixture of two diastereomers. The ratio of the diastereomers, isolated by chromatography, was close to 1:1 based on HPLC analyses. All enzyme reactions were carried out at 25 °C in phosphate buffer (50 mM, pH 7.6) and monitored at 264 nm by the decrease of the absorbance of the substrate. The amount of enzyme was chosen so that the decrease in absorbance was constant in the first 10 min. Briefly, 100 μL of enzyme was incubated with 800 μL of inhibitor solution at various concentrations for 10 min. The reaction was started by adding 100 μL of an 0.66 mM solution of adenosine. To determine $[I]/[S]_{0.5}$, v_0/v was plotted versus [I]/[S], where v_0 is the initial velocity of the uninhibited reaction, v is the initial velocity of inhibited reaction at various inhibitor concentrations, and [I] and [S] are the concentration of inhibitor and substrate, respectively. For each plot, the reaction was carried out in triplicate at five different inhibitor concentrations. The average initial velocity was then used in the aforementioned plots. Results normalized to the EHNA diastereomeric inhibitors are presented in Table 2.

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