Modified-Peptide Inhibitors of Amyloid β-Peptide Polymerization

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ABSTRACT: Cellular toxicity resulting from nucleation-dependent polymerization of amyloid β-peptide (Aβ) is considered to be a major and possibly the primary component of Alzheimer's disease (AD). Inhibition of Aβ polymerization has thus been identified as a target for the development of therapeutic agents for the treatment of AD. The intrinsic affinity of Aβ for itself suggested that Aβ-specific interactions could be adapted to the development of compounds that would bind to Aβ and prevent it from polymerizing. Aβ-derived peptides of fifteen residues were found to be inhibitory of Aβ polymerization. The activity of these peptides was subsequently enhanced through modification of their amino termini with specific organic reagents. Additional series of compounds prepared to probe structural requirements for activity allowed reduction of the size of the inhibitors and optimization of the Aβ-derived peptide portion to afford a lead compound, cholyl-Leu-Val-Phe-Phe-Ala-OH (PPI-368), with potent polymerization inhibitory activity but limited biochemical stability. The corresponding all-D-amino acyl analogue peptide acid (PPI-433) and amide (PPI-457) retained inhibitory activity and were both stable in monkey cerebrospinal fluid for 24 h.

Alzheimer's disease (AD)[1] is a progressive degenerative disease of the brain characterized by loss of cognitive function (dementia), and selective neuronal death, and the abnormal formation in the brain of neuritic amyloid plaques. In the United States alone, an estimated four million people are afflicted with AD, and the total annual cost associated with the care of these patients is at least *$100 billion* (*1*). Genetic, neuropathologic, and transgenic modeling studies implicate the accumulation of amyloid β-peptide (Aβ) as an important step in the pathogenesis of AD (*2*). Aβ is an approximately 40-residue proteolytic fragment of amyloid precursor protein (APP), a ubiquitously expressed transmembrane protein with an as yet undefined function (*3*). Under pathogenic conditions, Aβ polymerizes into extended β-sheet structures that result in the Aβ fibrils characteristic of amyloid plaque. While monomeric Aβ appears to be a rather innocuous molecule produced during normal metabolism (*4*, *5*), as it assembles into the structures that eventually produce fibrils it becomes extremely toxic to neuronal cells (*6*−*8*). The mechanism by which toxicity occurs appears to involve oxidative stress and microglial activation (*9*−*11*, and references therein). Suppression or prevention of this transition from monomeric to oligomeric and polymeric Aβ species has thus emerged as a goal in the development of a therapy for AD (*2*, *3*, *12*−*14*).

In this report we discuss our strategy for the development of inhibitors of Aβ polymerization. The nucleation-dependent polymerization mechanism of Aβ fibrillogenesis (*15*−*17*) suggests that even a modest decrease in the concentration of plaque-competent Aβ can have major benefits with respect to fibrillogenesis and plaque growth. Our general goal was to develop compounds that would bind to Aβ and, by doing so, introduce conformational or steric effects that would interfere with amyloidogenic Aβ−Aβ interactions. Because Aβ has such a high affinity for itself and the nature of these interactions has begun to be elucidated (*18*−*25*), we chose to use Aβ as the starting point for developing leads for inhibition of its polymerization.

MATERIAL AND METHODS

General. Synthesis reagents and solvents were obtained from commercial suppliers. Aβ_{1-40} was purchased from Bachem (Torrance, CA). D-(+)-Biotin, fluoresceinisothiocyanate (isomer I), and 5-(and-6)-fluoresceincarboxylic acid succinimidyl ester were from Molecular Probes, Inc. *N*-Acetylneuraminic acid and 2-iminobiotin succinimidyl ester were from Calbiochem. Cholic acid, *trans*-4-cotininecarboxylic acid, 2-imino-1-imadazolidineacetic acid, (−)-2-oxo-4-thiazolidinecarboxylic acid, (−)-menthoxyacetic acid, 2-norbornaneacetic acid, γ-oxo-5-acenaphthenebutyric acid, 2-thiophenesulfonyl chloride, and thiopheneacetyl chloride were from Aldrich.

Peptide Synthesis. Peptides were synthesized primarily by standard solid-phase techniques using Fmoc-based chemistry and either HBTU-based or DIC/HOBt coupling strategies. Automated syntheses were performed using an ACT 396 Multiple Peptide Synthesizer (Advanced ChemTech Inc., Louisville, KY) or a PS3 synthesizer (Rainin Instrument Co., Inc., Woburn, MA). Deprotected peptides cleaved from resin were purified by reverse-phase HPLC on C_{18} chromatography

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[1] Abbreviations: Aβ, amyloid β-peptide; AD, Alzheimer's disease; BUI, brain uptake index; CSF, cerebrospinal fluid; DIC, diisopropylcarbodiimide; DIEA, diisopropylethylamine; DMF, dimethylformamide; HATU, *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HBTU, *O*-Benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HOBt, *N*-hydroxybenzotriazole; HPLC, high-pressure liquid chromatography; DMSO, dimethyl sulfoxide. Standard three-letter and single-letter amino acid abbreviations are used throughout, with lower-case single letters indicating D-isomers.

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columns using acetonitrile−water−trifluoroacetic acid gradients. Analytical HPLC was performed similarly. Confirmations of syntheses were performed by the observation of the expected parent ion by electrospray mass spectrometry (ES-MS) performed on a Perkin-Elmer Sciex API I instrument at SynPep Corporation (Dublin, CA).

Modified Peptides. Amino terminal modifications of peptides were performed via modification of resin-bound peptide using standard peptide coupling conditions or direct coupling via acid chloride derivatives in the presence of a tertiary amine base: fluoresceinaminothiocarbonyl derivatives were prepared by reaction with fluoresceinisothiocyanate; fluoresceincarbonyl, biotinyl, and 2-iminobiotinyl derivatives were prepared from the corresponding *N*-hydroxysuccinimide active esters; *N*-acetylneuraminyl, cholyl, *trans*-4-cotininecarbonyl, 2-imino-1-imadazolidineacetyl, (−)-menthoxyacetyl, 2-norbornaneacetyl, γ-oxo-5-acenaphthenebutyryl, and (−)-2-oxo-4-thiazolidinecarbonyl derivatives were prepared by HBTU-mediated or similar coupling of the precursor carboxylic acids; and thiopheneacetyl and 2-thiophenesulfonyl derivatives were prepared from the corresponding acid chlorides.

Radiolabeling. The following preparation of ^{14}C-labeled **87** (cholyl-lvffa-NH_2, see Table 1 and Figure 3) is representative. A solution of 2.1 μmol of ^{14}C-labeled cholic acid (New England Nuclear, specific activity 48.6 mCi/mmol, 2 mM in ethanol) was concentrated at reduced pressure and treated with a solution of HATU (0.88 mg, 1.1 equiv) and DIEA (0.74 μL, 2.1 equiv) in DMF (50 μL). After 5 min, D-Leu-D-Val-D-Phe-D-Phe-D-Ala-amide (3.0 mg, 2.0 equiv.) was added, and the solution was stirred for 6 h at room temperature. Incorporation of the labeled cholic acid appeared complete by analytical HPLC after ~2 h. The crude reaction mixture was diluted into ~1 mL of aqueous acetonitrile and was purified by semipreparative reverse-phase HPLC (Zorbax C8 column, 80 °C, 10−85% solvent B over 50 min, where solvent A is 0.1% TFA in water and B is 0.085% TFA/1.2% water in acetonitrile). The yield of purified ^{14}C-PPI-457 was 55 μCi (~55%).

Inhibition Assay. Synthetic materials were assayed for inhibition of Aβ_{1-40} polymerization using an adaptation of the assay of Lansbury (*17*). Aβ was dissolved in DMSO as a 20× stock prior to dilution into buffer (100 mM NaCl, 10 mM sodium phosphate, pH 7.4) at a final concentration of 50 or 5 μM in a final volume of 250 μL in 96-well titer plates. Inhibitors were added as concentrated DMSO solutions. Plates were rotary shaken using a titer plate shaker (Lab-Line, Model 4625) at a rate of 500−800 rpm as determined with a tachometer. Progress of Aβ polymerization was monitored by measuring turbidity (*17*) as the apparent UV absorbance (for 50 μM Aβ mixtures) in a Bio-Rad Model 450 Microplate Reader equipped with a 405 nm filter. Alternatively, polymerization was monitored by measurement of the fluorescence of a 2 μL aliquot diluted into 400 μL of buffer (50 μM potassium phosphate, pH 7.5) containing 10 μM thioflavin-T (*26*, *27*). The fluorescence of each sample, expressed in arbitrary units, was read in a Hitachi F-4500 fluorescence spectrophotometer (λ_{ex}= 450 nm, λ_{em}= 482 nm).

Stability Assay in Cerebrospinal Fluid. Compounds **77** (PPI-368), **86** (PPI-433), and **87** (PPI-457) were dissolved in DMSO at 2, 2, and 0.75 mM and filtered through a 0.2 μm Nylon syringe filter, respectively. For each solution of compound, at 37 °C, 225 μL of Rhesus monkey CSF (Northern Biomedical Research, Muskegon, MI) was combined with 69 μL of phosphate-buffered saline (136 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 4.2 mM KH_2PO_4, pH 7.2) followed by 6 μL of DMSO solution in a polypropylene microcentrifuge tube, and the resulting mixtures were briefly vortexed. Final concentrations in 300 μL total volumes were 40, 40, and 15 μM for **77**, **86**, and **87**, respectively. Tubes were analyzed immediately or maintained at 37 °C for 24 h.

Analysis for intact compound was performed using a Hewlett-Packard 1090 series II HPLC equipped with a C4 Vydac #214TP51 1 mm diameter 250 mm long column with 5 μm particle size reverse-phase packing. Mobile phases were (A) 0.1% TFA in water, and (B) 0.085% TFA in acetonitrile−water (99:1 v/v). A solvent gradient of 10% B for 5 min followed by a linear gradient to 70% B over 60 min at a flow rate of 50 μL/min was used. Injection volumes were 100 μL for **77** and **86** and 250 μL for **87**. UV absorbance at 214 nm was monitored.

Brain Uptake Studies. The brain uptake index (BUI) of inhibitors was measured in the rat using the technique of Oldendorf (*28*). In this model the BUI is a measurement of the ability of a test compound to cross the blood brain barrier expressed as a percentage of that observed for a freely diffusible reference (tritiated water). Radio-labeled compounds were administered as a rapid bolus (200 μL) into the left common carotid artery (with the left external carotid artery ligated) of ketamine/xylazine-anesthetized male Sprague−Dawley rats (250−350 g). The animals were sacrificed 15 s later, and the amount of radioactivity within the ipsilateral forebrain was determined and the BUI computed using the equation below.

$$\text{BUI} = [(\text{dpm of test compound in brain})/(\text{dpm of water in brain})]/[(\text{dpm of test compound in injectate})/(\text{dpm of water in injectate})]$$

Biodistribution Studies. The biodistribution of compounds **87**, **88**, **89**, and **80** (cholic acid) was determined 10 min following an intravenous bolus in male Sprague−Dawley rats (250−350 g: n = 2−4/compound). Briefly, under ketamine/xylazine anesthesia, ^{14}C-labeled compounds (^{14}C-cholyl or ^{14}C-acetyl) were administered as a rapid (1−2 s) intravenous bolus via the left jugular vein (~50 μg/kg at 3 μCi/kg). At 10 min post administration the animals were sacrificed and organs of interest rapidly removed. The amount of radioactivity in each organ was determined from representative samples of each organ (~100 mg) by scintillation counting.

RESULTS

The assay used to evaluate potential inhibitors of Aβ is based on agitating a solution of Aβ_{1-40} and monitoring the solution for the formation of polymerizing peptide. Characteristic of a nucleation-dependent polymerization, the resulting curve of polymer formation with time is sigmoidal, having an inital flat or *lag* phase followed by a rapid *polymerization* phase and finished by a *plateau* (Figure 1).

Table 1.

compound number[a]	description[b]	position[c]	lag[d]	inhibition[d] (%)	[inhibitor][e] (μM)
	(A) Amyloid β-Peptide Sequences				
1	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV	Aβ(1−40)			
2	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA	Aβ(1−42)			
	(B) 50 μM Aβ Assay Data				
3	Aβ(1−40)[F19T, F20T]	Aβ(1−40)	1.8	0	
4	Aβ(1−40)[M35Nle]	Aβ(1−40)	1.6	0	
5	Aβ(1−40)[L34A, M35A]	Aβ(1−40)	1.4	0	
6	Aβ(1−40)[S8A, F19T, F20T, L34A, M35A]	Aβ(1−40)	1.1	0	
7	HDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV	Aβ(6−40)	1.9	0	
8	DAEFREVHHQKLVFFAEDVGSNKGAIIGLMVGGVV	Aβ(1−5,11−40)	1.9	33	
9	DAEFRHDSGYKLVFFAEDVGSNKGAIIGLMVGGVV	Aβ(1−10,16−40)	1.2	0	
10	DAEFRHDSGYEVHHQAEDVGSNKGAIIGLMVGGVV	Aβ(1−15,21−40)	1.55	0	
11	DAEFRHDSGYEVHHQKLVFFSNKGAIIGLMVGGVV	Aβ(1−20,26−40)	2.37	0	
12	DAEFRHDSGYEVHHQKLVFFAEDVGIIGLMVGGVV	Aβ(1−25,31−40)	1.58	0	
13	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAVGGVV	Aβ(1−30,36−40)	1	0	
14	(EVHHHHQQK)-KLVFFAEDVGSNKGAIIGLMVGGVV	Aβ(16−40)	> 4	100	
15	DAEFRHDSGYEVHHQ	Aβ(1−15)	1.4	80	
16	HDSGYEVHHQKLVFF	Aβ(6−20)	1.66	76	
17	EVHHQKLVFFAEDVG	Aβ(11−25)	1	47	
18	KLVFFAEDVGSNKGA	Aβ(16−30)	>4.5	100	
19	AEDVGSNKGAIIGLM	Aβ(21−35)	1	0	
20	SNKGAIIGLMVGGVV	Aβ(26−40)	0.8	0	
21	H-(DAEFRHDSGYEVHHQ)-NH_2	Aβ(1−15)	1	0	
22	acetyl-(HDSGYEVHHQKLVFF)-NH_2	Aβ(6−20)	1	0	
23	acetyl-(KLVFFAEDVGSNKGA)-NH_2	Aβ(16−30)	0.9	2	
24	(2-iminobiotinyl)-Aβ(1−40)-OH	Aβ(1−40)	2.3	0	
25	(*N*-acetylneuraminyl)-Aβ(1−40)-OH	Aβ(1−40)	2.8	31	
26	(fluoresceinaminothiocarbonyl)-Aβ(1−40)-OH	Aβ(1−40)	2.3	43	
27	(fluoresceincarbonyl)-Aβ(1−40)-OH	Aβ(1−40)	1.9	4	
28	biotinyl-(DAEFRHDSGYEVHHQ)-OH	Aβ(1−15)	1	0	
29	2-iminobiotinyl-(DAEFRHDSGYEVHHQ)-OH	Aβ(1−15)	1.2	0	
30	*N*-acetylneuraminyl-(DAEFRHDSGYEVHHQ)-OH	Aβ(1−15)	1	0	
31	cholyl-(DAEFRHDSGYEVHHQ)-OH	Aβ(1−15)	>4	100	
32	*trans*−4-cotininecarbonyl-(DAEFRHDSGYEVHHQ)-OH	Aβ(1−15)	1	0	
33	2-imino-1-imidazolidineacetyl-(DAEFRHDSGYEVHHQ)-OH	Aβ(1−15)	1.4	70	
34	(−)-menthoxyacetyl-(DAEFRHDSGYEVHHQ)-OH	Aβ(1−15)	1	0	
35	2-norbornaneacetyl-(DAEFRHDSGYEVHHQ)-OH	Aβ(1−15)	1	0	
36	γ-oxo-5-acenaphthenebutyryl-(DAEFRHDSGYEVHHQ)-OH	Aβ(1−15)	1	0	
37	(−)-2-oxo-4-thiazolidinecarbonyl-(DAEFRHDSGYEVHHQ)-OH	Aβ(1−15)	1	0	
38	thiopheneacetyl-(DAEFRHDSGYEVHHQ)-OH	Aβ(1−15)-OH)	1	0	
39	2-thiophenesulfonyl-(DAEFRHDSGYEVHHQ)-OH	Aβ(1−15)	1	0	
40	fluoresceincarbonyl-(DAEFRHDSGYEVHHQ)-OH	Aβ(1−15)	1	30	
41	cholyl-(HDSGYEVHHQKLVFF)-OH	Aβ(6−20)	>4.5	100	
			2	43	5
42	cholyl-(EVHHQKLVFFAEDVG)-OH	Aβ(11−25)	1.5	0	
43	cholyl-(KLVFFAEDVGSNKGA)-OH	Aβ(16−30)	>4.5	100	
44	cholyl-(AEDVGSNKGAIIGLM)-OH	Aβ(21−35)	1.6	51	
45	cholyl-(SNKGAIIGLMVGGVV)-OH	Aβ(26−40)	>4.5	87	
46	*N*-acetylneuraminyl-(HDSGYEVHHQKLVFF)-OH	Aβ(6−20)	1.3	64	
47	*N*-acetylneuraminyl-(EVHHQKLVFFAEDVG)-OH	Aβ(11−25)	1.3	77	
48	*N*-acetylneuraminyl-(KLVFFAEDVGSNKGA)-OH	Aβ(16−30)	1	0	
49	*N*-acetylneuraminyl-(AEDVGSNKGAIIGLM)-OH	Aβ(21−35)	1	53	
50	*N*-acetylneuraminyl-(SNKGAIIGLMVGGVV)-OH	Aβ(26−40)	1.3	0	
51	2-iminobiotinyl-(HDSGYEVHHQKLVFF)-OH	Aβ(6−20)	1.6	44	
52	2-iminobiotinyl-(EVHHQKLVFFAEDVG)-OH	Aβ(11−25)	1.2	40	
53	2-iminobiotinyl-(KLVFFAEDVGSNKGA)-OH	Aβ(16−30)	1.2	55	
54	2-iminobiotinyl-(AEDVGSNKGAIIGLM)-OH	Aβ(21−35)	1	66	
55	2-iminobiotinyl-(SNKGAIIGLMVGGVV)-OH	Aβ(26−40)	2.3	0	
56	fluoresceincarbonyl-(HDSGYEVHHQKLVFF)-OH	Aβ(6−20)	1.9	0	
57	fluoresceincarbonyl-(EVHHQKLVFFAEDVG)-OH	Aβ(11−25)	1.7	34	
58	fluoresceincarbonyl-(KLVFFAEDVGSNKGA)-OH	Aβ(16−30)	1.6	59	
59	fluoresceincarbonyl-(AEDVGSNKGAIIGLM)-OH	Aβ(21−35)	1.2	25	
60	fluoresceincarbonyl-(SNKGAIIGLMVGGVV)-OH	Aβ(26−40)	2	75	
61	cholyl-(DSGYEVHHQKLVFF)-OH	Aβ(7−20)	>4.5	100	
			2	0	16
62	cholyl-(SGYEVHHQKLVFF)-OH	Aβ(8−20)	1.5	0	
			2.1	0	16
63	cholyl-(GYEVHHQKLVFF)-OH	Aβ(9−20)	2.0	0	16
64	cholyl-(YEVHHQKLVFF)-OH	Aβ(10−20)	2.1	0	16
65	cholyl-(EVHHQKLVFF)-OH	Aβ(11−20)	1.5	0	16
66	cholyl-(VHHQKLVFF)-OH	Aβ(12−20)	2.1	0	16
67	cholyl-(HHQKLVFF)-OH	Aβ(13−20)	2.6	28	16

Table 1 (Continued)

compound number[a]	description[b]	position[c]	lag[d]	inhibition[d] (%)	[inhibitor][e] (μM)
68	cholyl-(HQKLVFF)-OH	Aβ(14−20)	2.0	49	16
69	cholyl-(QKLVFF)-OH	Aβ(15−20)	>5.0	100	
			2.3	33	5
70	cholyl-(KLVFF)-OH	Aβ(16−20)	>5.0	100	
			2.9	38	5
71	cholyl-(HDSGYEVHHQKLVFF)-NH_2	Aβ(6−20)	1.8	0	16
72	cholyl-(HDSGYEVHHQKLVF)-NH_2	Aβ(6−19)	1.4	0	16
73	cholyl-(HDSGYEVHHQKL)-NH_2	Aβ(6−17)	1.8	0	16
74	cholyl-(HDSGYEVHH)-NH_2	Aβ(6−14)	1.0	29	16
75	cholyl-(HDSGY)-NH_2	Aβ(6−10)	1.1	13	16
76	cholyl-(QKLVF)-OH	Aβ(15−19)	3.1	0	
			1.6	0	5
77	cholyl-(LVFFA)-OH (PPI-368)[f]	Aβ(17−21)	>5.0	100	
			1.6	33	5
78	iminobiotinyl-(KLVFF)-OH	Aβ(16−20)	1.6	90	
79	*N*-acetylneuraminyl-(KLVFF)-OH	Aβ(16−20)	0.9	11	
80	cholic acid		1	~0	
81	KLVFF	Aβ(16−20)	1.1	0	
82	cholyl-(AAAAA)-OH		0.8	0	
83	cholyl-(FKFVL)-OH		1.4	32	
84	cholyl-(KLTFF)-OH		1.7	0	
	(C) 5 μM Aβ Assay Data				
85	cholyl-(LVFFA)-NH_2 (PPI-382)[f]		12	100	
			10	63	1.6
			3.0	0	0.6
86	cholyl-(lvffa)-OH (PPI-433)[f]		>10	100	
			>10	100	2
87	cholyl-(lvffa)-NH_2 (PPI-457)[f]		>10	100	
			>10	100	1.6
			2.6	0	0.6
88	cholyl-(lvff)-NH_2 (PPI-458)[f]		4.0	65	
			4.0	53	1.6
			1.9	0	0.6
89	acetyl-(lvff)-NH_2		1.4	44	

[a] (A) Sequences of Aβ_{1-40} and Aβ_{1-42}. (B) List of experimental compounds assayed against 50 μM Aβ_{1-40}. (C) List of experimental compounds assayed against 5 μM Aβ_{1-40}. [b] Peptide sequences are written from amino to carboxyl termini. Unless otherwise noted, peptides are free amine acids. [c] Position denotes a sequence relative to Aβ_{1-40} (compound **1**). Modifications of peptides are at the peptidyl terminal α-amino group, and the carboxyl terminal moiety is indicated as acid (OH) or amide (NH_2). [d] Activities expressed as lag and percent inhibition are as described in the text and Figure 1. [e] Assays were generally performed at a mole ratio of inhibitor to Aβ_{1-40} of 1:1 (50 or 5 μM in parts B and C, respectively) unless a lower concentration of inhibitor is noted to the right of the assay data. [f] "PPI-xxx" designations refer to the entry numbers of these compounds in our laboratory's compound index.

An inhibitor changes the shape of the curve in three ways. If it interferes with nucleation, the lag time will increase. Inhibition of the polymerization during addition to fibril nuclei or short fibrils is expected to slow the rate of polymerization. Inhibition by removing Aβ from the polymerization pathway should reduce the plateau level reached. Combination effects can be expected as well and are observed (see below). The molecular level details of Aβ structure before, during, and after polymerization are still poorly defined, but once Aβ forms a dimer or higher oligomer, it is expected to have a high proportion of β-structure. Thus, inhibitors that bind well to smaller oligomers and perhaps even monomer may reasonably be expected to perturb fibrillogenesis at all stages.

The first compounds that we studied re-examined prior reports relating to Aβ−Aβ interactions and fibrillogenesis inhibition. The phenylalanylphenylalanyl dipeptide at position 19−20 has been previously identified as contributing to aggregation, and substitutions of this sequence have been reported to result in inhibitors of aggregation (*24*). We re-examined this report by synthesizing and assaying the FF → TT analogue **3** previously described. This peptide did indeed delay polymerization of Aβ_{1-40} but did not prevent eventual complete polymerization (Table 1).

Oxidation of Met-35 to the sulfoxide has been reported to increase aggregation (*21*). To prevent the possibility of oxidation, we prepared the isosteric norleucyl-35 analogue **4** and tested it as an inhibitor. As with compound **3**, a delay in lag was observed but full polymerization was eventually observed (Table 1).

Two other peptides were synthesized to test hypotheses regarding the role of different amino acyl residues in amyloidogenesis. Hydrophobic clustering in the carboxyl terminal domain of Aβ has been suggested to be energetically important in fibrillogenesis (*29*). Substitution of Leu-Met at position 34−35 with Ala-Ala was performed in **5** to investigate this domain. Sequence analysis using the GOR algorithm (*30*) suggested that changing Ser-8 to alanine would enhance the helical propensity of the amino terminal region of Aβ at the expense of a propensity for forming a β-turn. Combination of this substitution with the substitutions in peptides **3** and **5** was explored in peptide **6** (Aβ_{1-40}[S8A, F19T, F20T, L34A, M35A]). Both of these peptides uniformly demonstrated mild inhibitory properties, increasing

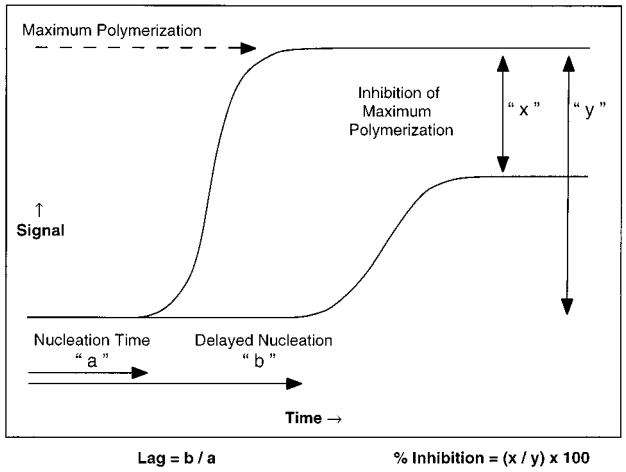

FIGURE 1: Schematic of an Aβ polymerization assay. A nucleated polymerization reaction is characterized by a period during which nuclei are formed but polymerization has yet to be observed (Nucleation Time). Once nucleation of polymerization has occurred, a rapid polymerization takes place resulting in a terminal level of polymer (Maximum Polymerization). Inhibition can be observed as prolongation of the period before polymerization accelerates (Delayed Nucleation), reduction in the level of polymer formed (Inhibition of Maximum Polymerization), slowing of the rate of polymerization once initiated, or a combination of these effects. Numerical values are calculated from these data as "Lag", the proportional increase in nucleation time, and as "Inhibition", the percent reduction of maximum polymerization in the absence of inhibitor.

the lag period by less than a factor of 2, but failed to prevent eventual full aggregation within a short time period.

An alternative strategy for disrupting Aβ polymerization was to introduce deletions into Aβ. This approach was explored with a pentapeptide deletion scan in peptides **7**−**13**. These compounds exhibited mild to moderate inhibition of polymerization, comparable to the activities of peptides **3**−**6**. Interestingly, a compound in which the polar residues of the HHQK region of positions 13−16 were each doubled, **14**, was a very potent inhibitor. Because of its large size this lead was not explored further at this time.

In an effort to identify smaller portions of the Aβ sequence with inhibitory activity, we scanned the Aβ sequence in 15-residue sections in compounds **15**−**23**. Of these peptides, the free amine acid of Aβ(16−30), **18**, was found to be very potent, preventing polymerization for the time course of the assay to a lag of >4.5.

In an effort to enhance the inhibitory activities of Aβ-derived peptides, we began to study the effect of modifying peptides with organic reagents (Figure 2). Initially Aβ_{1-40} was modified, in part to evaluate the effects of introducing tagging moieties for assay development. These compounds, **24**−**27**, showed modestly higher potencies regarding increases in lag and began to reduce the total amount of polymer detected. This strategy of *N*-terminal modification was next extended to Aβ-derived pentadecapeptides.

In this series of compounds, **28**−**41**, a variety of *N*-terminal modifying reagents were examined on Aβ_{1-15} to explore a range of pharmacophoric variables including polarity, charge, hydrophobicity, and shape. From these compounds, the modifiers cholic acid, *N*-acetylneuraminic acid, 2-iminobiotin, and fluoresceincarboxylic acid were selected to study as modifiers of additional Aβ pentadecapeptides in compounds **42**−**60**. From these two series of compounds, the cholyl group, modifying Aβ_{1-15}, Aβ_{6-20}, and Aβ_{16-30}, provided inhibitors of Aβ polymerization that were of the same or greater potency than the previously identified potent inhibitors. The resulting compounds, while potent, were still higher in molecular weight than is desirable in a robust lead compound.

The next two series of compounds were designed to determine if the cholylpentadecapeptide **41** could be reduced in size while preserving inhibitory activity. Cholyl-modified peptides truncated from the amino terminus, compounds **61**−**70**, and from the carboxyl terminus, compounds **71**−**75**, were prepared. As the core Aβ_{6-20} sequence was shortened from the amino terminus, potency was quickly lost in compounds **61**−**67**. Potency returned with the seven to five residue peptides **68**−**70**. Potency was lost on truncation at the carboxyl terminus and was not regained substantially in the

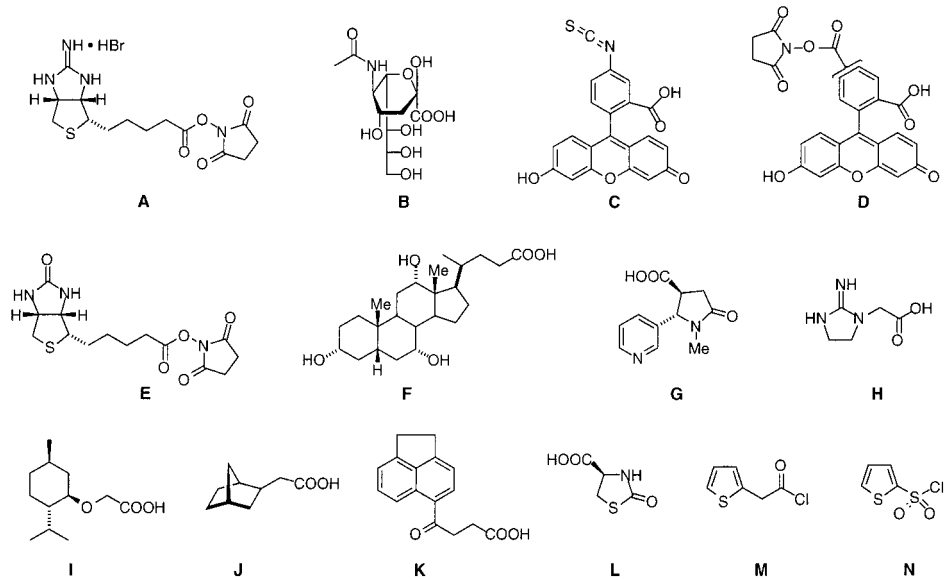

FIGURE 2: Reagents used in the *N*-terminal modification of peptides. Each lettered reagent was used for the synthesis of the compound numbers noted which correspond to Table 1: (A) **24**, **29**, **51**−**55**, **78**; (B) **25**, **30**, **46**−**50**, **79**; (C) **26**; (D) **27**, **40**, **56**−**60**; (E) **28**; (F) **31**, **41**−**45**, **61**−**77**, **80**, **82**−**88**; (G) **32**; (H) **33**; (I) **34**; (J) **35**; (K) **36**; (L) **37**; (M) **38**; (N) **39**.

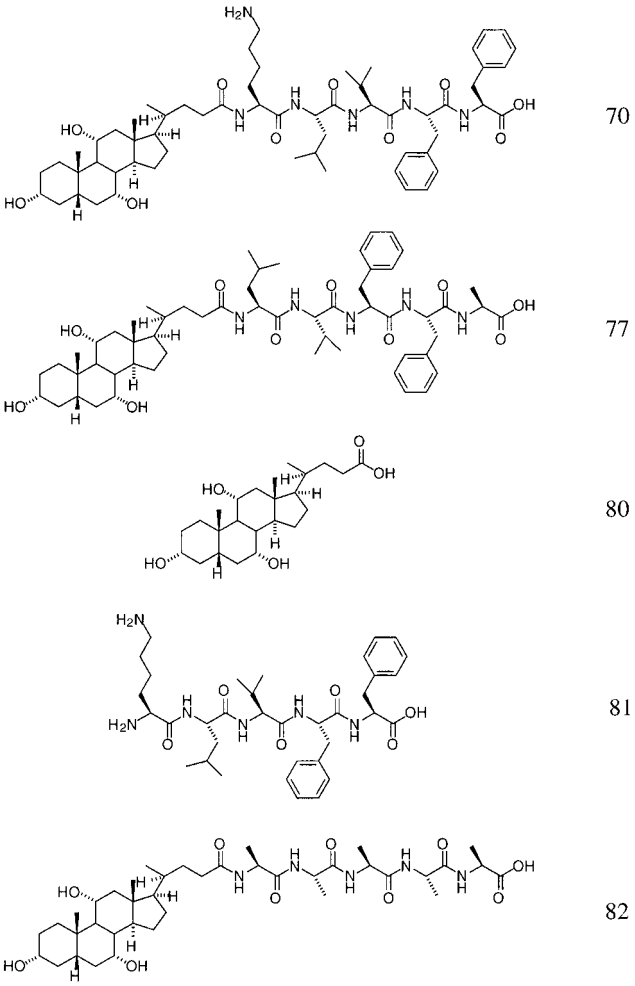

FIGURE 3: Structures of compounds **70**, **77**, **80**, **81**, and **82**.

shorter peptides. The carboxyl region of the initial lead, **41**, thus appeared to be associated with potency.

The structure−activity properties of the smallest and still highly potent inhibitors were further examined by synthesizing and assaying compounds **76**−**79**, in which the peptide was shifted one residue toward the amino or carboxyl terminus of the Aβ sequence or the *N*-terminal modification was changed. In compound **77** (Figure 3), high potency was retained (Figure 4), while obtaining a compound with only a single ionizable group and reduced polarity overall. This compound was of interest because of possibly enhanced blood-brain barrier permeability in comparison with other larger and more polar compounds.

The combination of both the peptide and the organic groups is required for the activity of **70** (Figure 4). Cholic acid alone, **80**, is inactive as is peptide alone, **81**. Replacement of the peptide LVFFA in **77** with pentaalanine, in **82**, abolishes activity. Minor rearrangement or substitutions within the peptide, in **83** and **84**, also dramatically reduce activity. Taken together, these data demonstrate a synergistic effect of a specific peptide in combination with a specific organic modifier providing potent inhibition of Aβ polymerization.

The value of **77** as a drug lead is expected to be limited because of the anticipated biological instability of the L-peptide moiety. As shown in Figure 5, **77** is unstable to Rhesus monkey CSF, being completely degraded within 24 h. The all-D analogue acid **86** and amide **87** (structures, Figure 6), however, are quite stable, showing little to no degradation in the same time period. The D-compounds, particularly the amide **87**, are insufficiently soluble to assay at 50 μM Aβ. The nucleated polymerization assay was therefore adapted to 5 μM Aβ(1−40) to compare these compounds. In this 5 μM assay both compounds are very potent inhibitors of polymerization (Figure 7). Further, both **86** and **87** are stable to CSF over 24 h (Figure 5). Substantial activity is maintained even when the terminal alanyl residue is deleted to obtain a modified tetrapeptide **88**. Even at submolar equivalents relative to Aβ, **87** and its analogues maintain potency (Table 1 and Figure 8). With the overall hydrophobic character of the *N*-acyl peptide, amides **87**, **88**, and **89**, it was expected that these compounds might have observable blood-brain barrier permeability. In fact, brain levels in excess of sucrose control levels were observed for the ^{14}C-labeled cholyl peptide amides **85**, **87**, and **88** (Table 2).

Table 2: Determination of Brain Uptake Index (BUI) in the Rat for Inhibitors Relative to Tritiated Water[a]

compound	n	% CNS vs total injected mean (sem)	BUI, percent mean (sem)
water[b,c]	13	5.958 (0.216)	100
sucrose[b,d]	11	0.047 (0.003)	0.78 (0.05)
cholic acid[b]	5	0.063 (0.005)	1.02 (0.09)
85[b]	6	0.110 (0.002)	1.79 (0.04)
87[b]	7	0.215 (0.023)	3.09 (0.34)
88[b]	6	0.216 (0.025)	4.25 (0.49)
89[e]	10	0.255 (0.050)	3.72 (0.53)

[a] Brain Uptake of Water Is Set at 100%. [b] Formulated in 50 mM hydroxypropyl-γ-cyclodextrin in PBS. [c] Positive control for brain uptake. [d] Negative control for brain uptake. [e] Formulated in 0.16% Tween-80/1.3% PEG-300/0.54% EtOH (v/v) in saline.

Table 3: Biodistribution in the Rat

	% in organ vs total injected (10 min post i.v. bolus injection)			
organ	**80**[a]	**87**[a]	**88**[a]	**89**[b]
lung	0.4	0.4	11	0.4
kidney	0.6	1.3	2.3	0.4
liver	10	22	25	34
duodenum	16	23	25	1
heart	0.2	0.1	0.1	0.1
blood	0.8	0.2	0.4	2.0

[a] Formulated in 50 mM hydroxypropyl-γ-cyclodextrin in PBS. [b] Formulated in 0.16% Tween-80/1.3% PEG-300/0.54% ethanol in saline.

A biodistribution study, however, revealed marked hepatic first-pass elimination for all compounds tested. The observation of similar profiles (Table 3) for cholic acid and the cholyl-modified compounds suggests that the cholyl-modified compounds are being eliminated as if they were endogenous bile components, producing the high duodenal levels. In comparison, the acetyl compound 89 appears to have a more favorable profile characterized by similar blood brain barrier permeability but with decreased duodenal clearance and greater plasma exposure. These observations indicate that this class of bile acid-modified peptides is unsuitable for further development to obtain a drug candidate.

DISCUSSION

The nucleation assay used as a primary screen in this work has shown itself to be a reliable assay, allowing identification

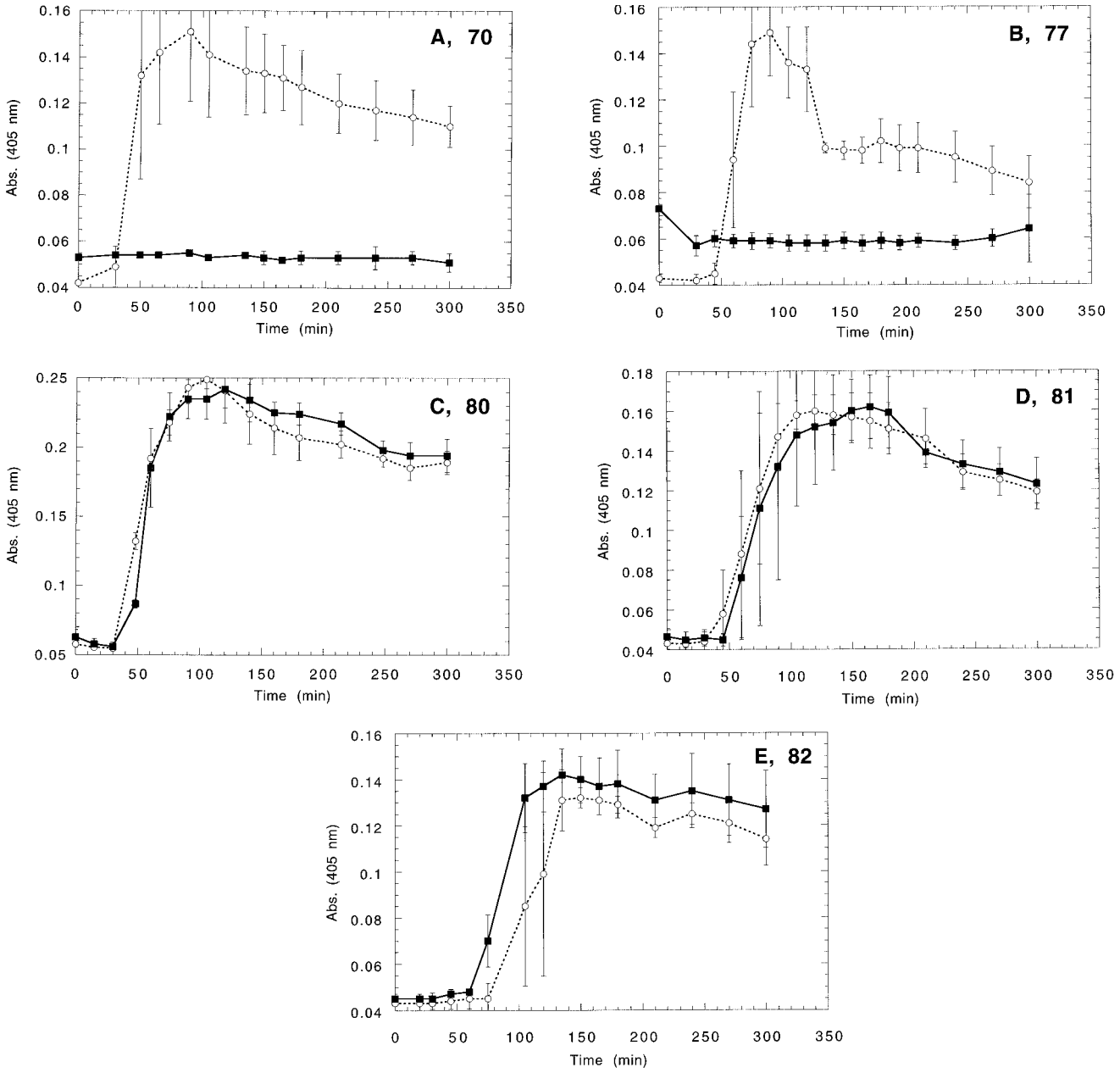

FIGURE 4: Assay at 50 μM Aβ_{1-40} (○) of compounds **70** (A), **77** (B), **80** (C), **81** (D), and **82** (E), each at 50 μM concentration (■). The potency of **70** results from a combination of the *N*-terminal organic group and the peptide moiety as shown by the low potency of **80** and **81**. The peptide side chains are required for activity as well, as indicated by the loss of activity in the all alanyl compound **82**.

of potent inhibitors of Aβ polymerization. Limitations of this assay do exist, however. The quality of the Aβ used in the assay must be closely monitored to ensure reproducible results. Several commercial suppliers can provide high quality material that performs appropriately in the assay. A "good" lot of Aβ can go "bad" with time, and appropriate care must be exercised. The very nature of the assay and the poorly understood mechanism of Aβ polymerization combined with the relatively high concentration of Aβ in the assay preclude direct measurement of a binding constant for the interaction of an inhibitor with Aβ. For routine screening we prefer to test compounds at a concentration equimolar to that of the Aβ. As can be seen in the work described here, low solubility of a test compound can make it difficult to assay accurately. For potent inhibitors, however, significant inhibition can be observed even at submolar equivalents of inhibitor relative to Aβ.

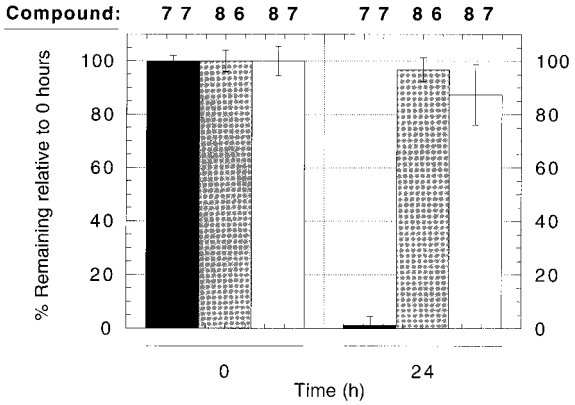

FIGURE 5: Stability in Rhesus monkey cerebrospinal fluid of compounds **77** and its all-D acid and amide analogues, **86** and **87**. After 24 h in CSF, L-aminoacyl **77** is completely degraded while the D-aminoacyl acid and amide compounds are intact.

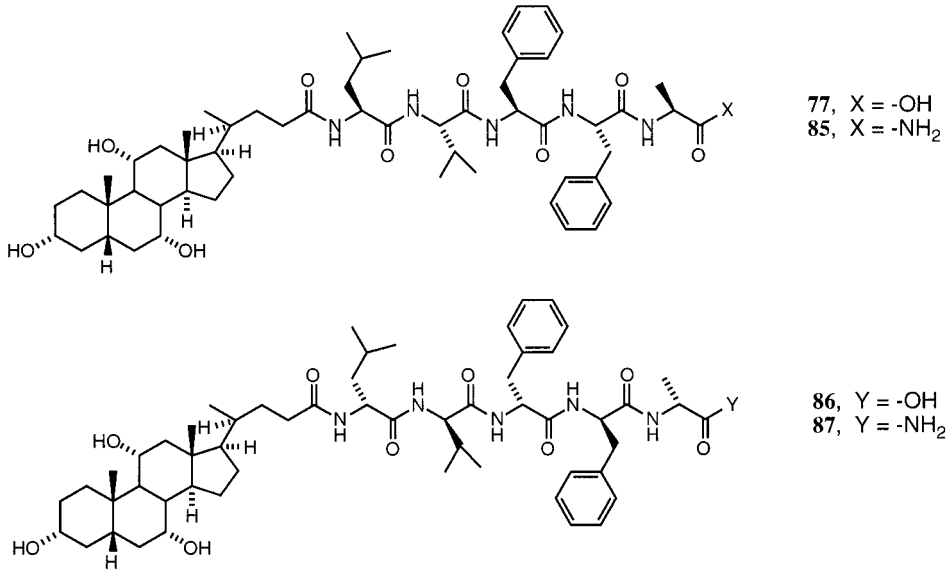

FIGURE 6: Structures of compounds **77**, **85**, **86**, and **87**.

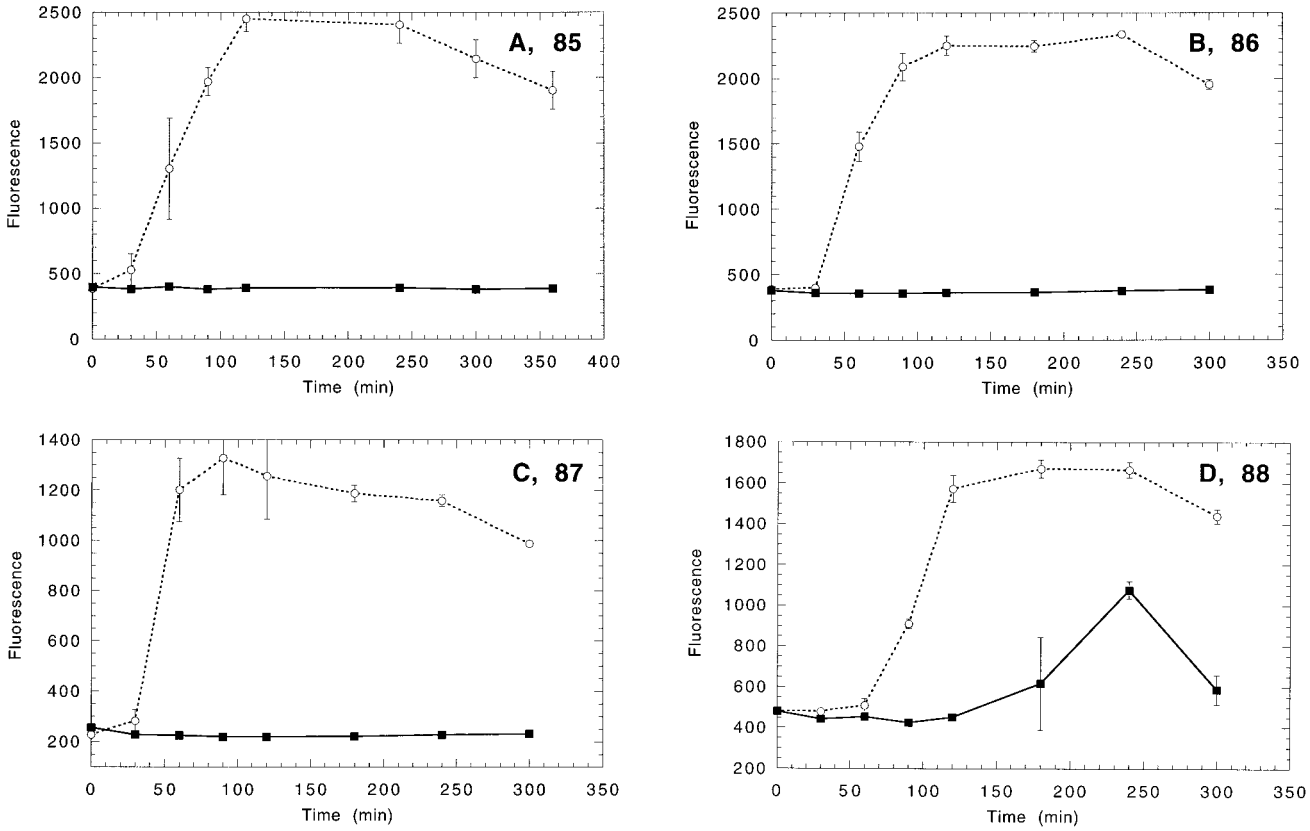

FIGURE 7: Assay at 5 μM Aβ_{1-40} (○) of compounds **85** (A), **86** (B), **87** (C), and **88** (D), each at 5 μM concentration (■).

Lowering the concentration of the Aβ in the assay makes observation of polymerization more difficult. Turbidity measurements become less practical below 10^{-5} M, and thioflavin-T binding loses utility below 10^{-6} M. Alternative assays to allow direct or indirect observation of inhibitors with Aβ at lower concentrations are possible using radiolabeled compounds in a displacement assay or by testing assay solutions directly on cells for the formation of toxic forms of Aβ.

During this work we have been able to obtain compounds of a range of sizes that are potent inhibitors of Aβ polymerization (e.g., **14**, **18**, **43**, and **77** and its analogues). With the goal of having an inhibitor of the smallest size while preserving high potency, we have not fully explored the properties of the larger peptide inhibitors. The ability of L-amino acid-based compounds such as **14** and **18** to have high potency suggests the possibility of therapeutic approaches based on gene expression. The practicality of such an approach remains to be examined.

The lower molecular weight compounds that have been identified during this work that are potent inhibitors of the polymerization of Aβ are of considerable interest as lead compounds for the development of therapeutic agents. The combination of an appropriate key pharmacophore and an

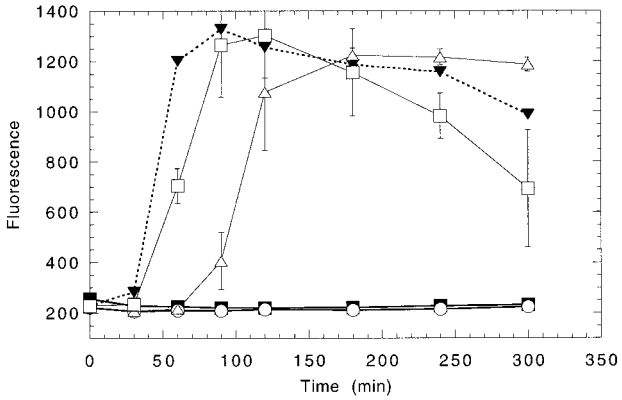

FIGURE 8: Dose response for compound **87** assayed against Aβ_{1-40}. Aβ_{1-40} alone at 5 μM (▼). Aβ_{1-40} at 5 μM in the presence of **87** at 0.2 μM (□), 0.6 μM (△), 1.7 μM (○), and 5 μM (■).

Aβ-specific recognition group results in a compound with greater activity than its constituent components. The undesirable hepatic accumulation is ascribed to the bulky bile acyl group, and efforts are underway to replace this group while preserving potency. The identification of all-D peptide-based compounds with high potency establishes a rather novel class of compounds. Further exploration of the structure−activity relationships and pharmacological properties of these compounds and their analogues is underway. More extensive characterization of one of these inhibitors, **77** (cholyl-LVFFA-OH), has shown that it inhibits polymerization of Aβ_{1-42} as well as Aβ_{1-40}, and that inhibition of polymerization correlates with inhibition of cellular toxicity (*31*).

Recent reports by others confirm the general observations of this work that the central hydrophobic region of Aβ comprised of the LVFFA sequence is particularly important for fibrillogenesis. Soto and co-workers have described several peptides based on the hydrophobic core sequence of Aβ that display antifibrillogenesis, particularly at molar excess (*32*). Tjernberg and co-workers have described a potent acetyl hexapeptide amide active at equimolar concentration to Aβ_{1-40} at 100 μM (*33*). The most potent inhibitors we have tested are of similar or greater potency.

The ability of inhibitors such as those described above to be of therapeutic utility remains to be determined. Halting the progression of familial amyloid polyneuropathy, which is caused by transthyretin amyloidosis, by liver transplantation (*34*, *35*) and the treatment of hemodialysis-related amyloidosis with renal transplatation (*35*) suggests that stopping the continued accumulation of β-amyloid polymerization in vivo may be therapeutic. Chemotherapy-based treatments of other amyloidoses (*35*, *36*) also suggest that neurotoxic forms of Aβ and their deposits may be controllable. AD amyloid plaques are observed to be porous and exhibit competing aggregation and disaggregation processes (*37*). Recently, diffusible nonfibrillar but highly neurotoxic forms of oligomeric Aβ_{1-42} with cell-surface binding properties have been characterized (*38*). Inhibition of oligomerization, and of continued plaque growth, would therefore be expected to allow natural clearance processes to reduce or eliminate plaque and other neurotoxic forms of Aβ. An associated reduction in pathological effects would be anticipated. The demonstration that modified peptides can be potent inhibitors of β-peptide polymerization opens the opportunity to explore further this type of compound for optimization of pharmacological properties and potential therapeutic use.

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