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Discovery of a Novel Pharmacological and Structural Class of Gamma Secretase Modulators Derived from the Extract of *Actaea racemosa*

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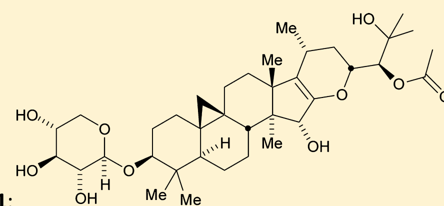
Supporting Information

ABSTRACT: A screen of a library of synthetic drugs and natural product extracts identified a botanical extract that modulates the processing of amyloid precursor protein (APP) in cultured cells to produce a lowered ratio of amyloid-beta peptide (1−42) (Aβ42) relative to Aβ40. This profile is of interest as a potential treatment for Alzheimer's disease. The extract, from the black cohosh plant (*Actaea racemosa*), was subjected to bioassay guided fractionation to isolate active components. Using a combination of normal-phase and reverse-phase chromatography, a novel triterpene monoglycoside, **1**, was isolated. This compound was found to have an IC_{50} of 100 nM for selectively reducing the production of amyloidogenic Aβ42 while having a much smaller effect on the production of Aβ40 (IC_{50} 6.3 μM) in cultured cells overexpressing APP. Using IP-MS methods, this compound was found to modulate the pool of total Aβ produced by reducing the proportion of Aβ42 while increasing the relative amounts of shorter and less amyloidogenic Aβ37 and Aβ39. Concentrations of **1** sufficient to lower levels of Aβ42 substantially (up to 10 μM) did not significantly affect the processing of Notch or other aspects of APP processing. When **1** (10 μg) was administered to CD-1 normal mice intracerebroventricularly, the level of Aβ42 in brain was reduced. Assays for off-target pharmacology and the absence of overt signs of toxicity in mice dosed with compound **1** suggest a comparatively selective pharmacology for this triterpenoid. Compound **1** represents a new lead for the development of potential treatments for Alzheimer's disease via modulation of gamma-secretase.

Compound **1**:



KEYWORDS: *Alzheimer's disease, amyloid-beta peptide, gamma-secretase modulator, botanical extract, Actaea racemosa, triterpene glycoside*

Alzheimer's disease (AD) is the most common type of dementia.[1] It is the sixth leading cause of death in the United States and the fifth leading cause of death among those aged 65 or older. Total societal costs in the US to care for those afflicted with this disease are in excess of \$200 billion per year. An estimated 5.4 million Americans currently have AD. As the population continues to age, this figure will continue to rise dramatically over the next several decades. The development of effective therapies that delay the onset and progression of this disease can reverse this trend. Such drugs, however, have yet to be approved. The lack of such disease-modifying therapies underscores the magnitude of this major unmet medical need. An evolving understanding of the molecular basis of this disease is guiding the development of therapies with which to treat it.[2−4]

While debate continues about the underlying causes of AD and how they relate to tractable molecular targets for the development of potentially effective therapies, the central role of amyloid-beta peptide (Aβ) in the onset and progression of disease appears to be firmly established, even if a full understanding of how Aβ contributes to the disease process remains to be achieved.[5] In particular, Aβ42 appears to be important even though it is a minor component of the total Aβ pool under normal conditions.[6] Genetics of early onset variants

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of AD,[7,8] biochemical studies of the toxicity of Aβ42,[9] and manipulations of ratios of Aβ(1−*x*) levels in animal models of AD[10,11] all consistently support the importance of the highly amyloidogenic Aβ42 in disease etiology and many of these same studies also suggest that lowering the proportion of Aβ42 relative to other shorter (and less amyloidogenic) forms of Aβ is a compelling approach to disrupt or halt the disease pathway.[6,12,13]

Recent failures of experimental drugs intended to be disease-modyfying therapies for AD raise questions about the validity of Aβ as a drug target and provide an impetus to reexamine how best to design clinical trials to test drugs that target Aβ.[14] The recent discovery of a mutation in the Aβ region of APP that lowers the production of Aβ by 20−40% and is protective against AD provides direct evidence that moderately lowered Aβ production in humans can be beneficial.[15] Whether safe and effective pharmacologically induced reduction of Aβ levels can prevent or slow cognitive decline in patients once the disease process is started remains to be determined. Despite our advances in understanding the cascade of AD, confirmation of the amyloid hypothesis still awaits a biochemically potent and pharmacodynamically effective experimental drug to show efficacy in appropriately designed human clinical trials.

Aβ is produced through the sequential action of proteases on amyloid precursor protein (APP), an integral transmembrane protein.[16] Beta-secretase cleaves the bulk of the cytosolic domain of APP resulting in the APP beta-C-terminal fragment (β-CTF), still present in the membrane. Subsequently, gamma-secretase (γ-Sec) cleaves β-CTF within the membrane-spanning sequence to release Aβ. It is at this point that Aβs varying in length at the C-terminus are created. Because the primary form of Aβ associated with the onset of AD is the longer Aβ42 and the enzyme directly responsible for its formation is γ-Sec, this enzyme emerged as a target for selective inhibition or modulation.[12]

Inhibitors of γ-Sec emerged early as a target for the development of potential therapeutics for AD with the goal of lowering levels of total Aβ production and subsequent accumulation in amyloid plaques, the histopathological hallmark of AD. Inhibition of γ-Sec is readily achieved with compounds that bind to presenilin, the component of the γ-Sec complex containing the enzyme active site.[17] Such γ-Sec inhibitors (GSIs) potently reduce total γ-Sec activity, and reduce the amount of total Aβ produced. In doing so, however, processing by γ-Sec of other of its substrates is also inhibited and unwanted side-effects result. The best known example of this effect is the negative consequence of inhibiting the γ-Sec-mediated processing of the protein Notch, a transmembrane receptor. γ-Sec-mediated cleavage of Notch releases Notch intracellular domain (NICD) which regulates gene expression during cellular growth. Inhibition of γ-Sec reduces production of NICD and results in toxicity to a variety of cell types. GSIs have been reported to cause abnormalities in brain, gastrointestinal tract, thymus, spleen, and skin in animals and in man,[13,18] and the overwhelming majority of GSIs have been halted at various stages of preclinical and clinical development. Even the Notch-sparing GSI avagacestat (BMS-708163) was reported to have significant side effects at higher doses, restricting its further development to lower doses.[19] Thus, a strategy to lower total Aβ levels with GSIs is challenged by undesirable side effects at doses required to lower Aβ levels. A preferred strategy that has emerged is to modulate APP processing so that production of Aβ42 is selectively lowered without compromising the processing of other essential substrates such as Notch.

The possibility that such γ-Sec modulators (GSMs) could exist was first reported by Golde and co-workers when they observed that some nonsteroidal anti-inflammatory drugs (NSAIDs) could selectively lower levels of Aβ42 without apparently affecting the action of γ-Sec on other aspects of APP processing or the processing of other substrates such as Notch.[20] The potency of these and related compounds as GSMs was limited however. Despite this significant limitation, tarenflurbil (the *R*-enantiomer of the NSAID flurbiprofen) proceeded into human clinical trials. Not surprisingly, no significant modification was observed of plasma Aβ42 levels[21] which undoubtedly contributed to its lack of efficacy in trials for the treatment of AD.[22] GSMs based on biaryl acetic acids and other heterocycles have been developed that have progressed toward or into clinical studies,[13] with any data on clinical efficacy yet to be announced.

A broader approach to identifying compounds with the desired type of selective activity was undertaken by one of our laboratories.[23] A highly diverse compound library comprised of a variety of substances of comparative safety based on prior use in humans as drugs, traditional medicines, or nutraceuticals was compiled, and, using a cell-based screening approach, this library of substances was screened specifically for selective lowering of the level of Aβ42 relative to Aβ40 while also not affecting cell viability. Interestingly, of the approximately 2000 samples assayed, only one provided a robust selective effect.[24] It was a sample of an extract of the native American medicinal plant black cohosh (*Actaea racemosa*, also commonly known by the alternative name *Cimicifuga racemosa*).

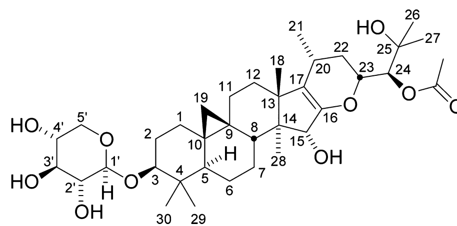
Black cohosh is well-known for its use in traditional folk medicine as an anti-inflammatory and for the treatment of hot flashes during menopause.[24−26] The pharmacology of the extract and several of its molecular components is relatively unstudied. The isolation and characterization of its constituents is similarly of relatively limited scope. Not surprisingly for a botanical extract, known major classes of compounds isolated from the extract of the roots and rhizomes of this plant include a variety of related triterpenes and their glycosides, and a group of phenolic carboxylic acids.[27] In this report, we describe our efforts to isolate and characterize compounds responsible for the ability of this extract to selectively reduce the production of Aβ42 via modulation of gamma-secretase, culminating in the identification of a structurally novel class of GSMs.

RESULTS AND DISCUSSION

Fractionation of Black Cohosh Extract. Raw extract was iteratively fractionated, using normal phase chromatography and reverse-phase high performance chromatography (RP-HPLC). Fractions obtained through this process were screened for activity using an ELISA for Aβ40 and Aβ42 in cell conditioned media to identify samples with activity to selectively lower levels of Aβ42. As a set of fractions were obtained and assayed, higher potency fractions were then selected for further separation.

Fractionation of 6.4 g of *Actaea racemosa* extract revealed large quantities of actein (**2**), a compound previously described from black cohosh,[28,29] which did not reduce Aβ42 in our cell-based assay. In addition, we isolated the cimigenols **3** (cimigenol-3-*O*-beta-D-xylopyranoside)[30] and **4** (cimigenol-3-*O*-alpha-D-arabinopyranoside),[30,31] which also were inactive. Most of the activity cofractionated with the known 24-*O*-

Table 1. NMR Spectroscopic Data for 24-*O*-Acetylhydroshengmanol 3-*O*-*β*-D-Xylopyranoside-$\Delta^{16,17}$-enol Ether (1)[a]



carbon no.	δ (ppm)	proton no.	δ (ppm)	J (Hz)	NOESY correlations	HMBC correlations
C-1	32.99	1-H_α	1.55	$J_{1\alpha,1\beta}$ = 13.5, $J_{1\alpha,2\beta}$ = 13, $J_{1\alpha,2\alpha}$ = 4		
		1-H_β	1.24	$J_{1\beta,2\alpha}$ = 3.6, $J_{1\beta,2\beta}$ = 3.6	19-H_α	
C-2	30.39	2-H_α	1.92	$J_{2\alpha,2\beta}$ = 13.5, $J_{2\alpha,3}$ = 4.2		
		2-H_β	1.67	$J_{2\beta,3}$ = 11.4		10
C-3	89.86	3-H	3.21		5-H	1′
C-4	41.88					
C-5	48.61	5-H	1.31	$J_{5,6\alpha}$ = 4, $J_{5,6\beta}$ = 12.7	3-H	
C-6	21.59	6-H_α	1.60	$J_{6\alpha,6\beta}$ = 12.7, $J_{6\alpha,7\alpha}$ = 13.5		
		6-H_β	0.84		19-H_β	
C-7	27.78	7-H_α	1.10		28-H	8
		7-H_β	1.62			
C-8	47.89	8-H	1.81	$J_{8,7\alpha}$ = 12, $J_{7,7\beta}$ = 3.7	15-H, 18-H, 19-H_β	8
C-9	20.58					
C-10	27.93					
C-11	27.07	11-H_α	2.12		28-H	13
		11-H_β	1.17		19-H_α	
C-12	30.59	12-H_α	1.94		21-H, 28-H	9
		12-H_β	1.60		18-H	
C-13	48.09					
C-14	50.20					
C-15	79.93	15-H	4.29		8-H, 18-H	8, 13, 16, 17, 28
C-16	150.26					
C-17	123.09					
C-18	1.21	18-H	24.48		8-H, 15-H, 12-H_β, 20-H	12, 13, 14, 17
C-19	32.76	19-H_α	0.33		1-H_β, 11-H_β	1, 5, 8, 9, 10, 11
		19-H_β	0.70		6-H_β, 8-H	1, 5, 8, 9, 10, 11
C-20	28.27	20-H	2.54	$J_{20,22\alpha}$ = 9.8, $J_{20,22\beta}$ = 5.3	18-H, H-23	16, 17
C-21	20.28	21-H	1.01		12-H_α	17, 20, 22
C-22	37.25	22-H_α	1.44	$J_{1\alpha,1\beta}$ = 13.5	21-H	
		22-H_β	1.77			17
C-23	77.19	23-H	4.19	$J_{23,22\alpha}$ = 12, $J_{23,22\beta}$ = 1.5	H-20	
C-24	79.90	24-H	4.87			25, 26, 27, $CH_3\underline{C}O$
C-25	73.09					
C-26	26.41	26-H	1.24			24, 25, 27
C-27	27.10	27-H	1.31			24, 25, 26
C-28	13.69	28-H	0.98		7-H_α, 11-H_α, 12-H_α	8, 13, 14, 15
C-29	15.27	29-H	0.88			3, 4, 5
C-30	25.83	30-H	1.04			3, 4, 5
C-1′	107.16	1′-H	4.27	$J_{1',2'}$ = 7.6		3
C-2′	75.30	2′-H	3.18	$J_{2',3'}$ = 9.1		
C-3′	77.81	3′-H	3.29	$J_{3',4'}$ = 9.1		
C-4′	71.15	4′-H	3.46	$J_{4',5a'}$ = 5.4, $J_{4',5b'}$ = 9.5		
C-5′	66.49	5′-H_a	3.81	$J_{5a',5b'}$ = 11.4		
		5′-H_b	3.17			
$\underline{CH_3}CO$	20.63	$C\underline{H_3}$	2.12			$\underline{C}H_3CO$
$CH_3\underline{C}O$	172.31					

[a] ^{1}H NMR, dqfCOSY, HMQC, HMBC, and NOESY spectra were acquired using a Varian INOVA 600 MHz spectrometer using methanol-d_4 as solvent. Chemical shifts were referenced to ($CD_2\underline{H}OD$) = 3.31 ppm and ($\underline{C}D_3OD$) = 49.05 ppm. Coupling constants were determined from dqfCOSY spectra.

acetylhydroshengmanol 3-*O*-beta-D-xylopyranoside (**5**).[32] Additional fractionation of fractions containing **5** led to isolation of a novel derivative of **5** in which the hemiketal present in **5** is dehydrated to create an enol ether: (24*S*)-24-*O*-acetylhydrosh-

engmanol 3-*O*-beta-D-xylopyranoside $\Delta^{16,17}$-enol ether, **1** (Table 1). Upon repeating isolation of **1**, an additional minor component was isolated that was identified as the C-24 epimer of **1**, compound **6**.

In addition, we isolated both the L-arabinosyl analogue **7**[33] of D-xylosyl shengmanol **5**, and the L-arabinosyl analogue **8** of D-xylosyl shengmanol enol ether **1**, as well as the C-24 epimer **9** of shengmanol xyloside **5**. Compound **7** was found to be less active than the corresponding xyloside analogue **5**, whereas compound **9** was modestly more potent than **5** (Table 2). Arabinoside **8** was significantly less active than the xyloside **1**.

Table 2. Names and IC_{50}'s for Reduction of Aβ40/42 of Compounds Isolated from Black Cohosh Extract (1−9) and Chemical Derivatives of 1 (10−12)

compd no.	compd name	IC_{50} (nM)	
		Aβ40	Aβ42
1	24-*O*-acetylhydroshengmanol (Δ-16,17)-enol ether-3-*O*-β-D-xylopyranoside, (24*S*)	6300	100
2	actein	>50 000	>50 000
3	cimigenol-3-*O*-β-D-xylopyranoside	>50 000	>50 000
4	cimigenol-3-*O*-α-L-arabinopyranoside	>50 000	>50 000
5	24-*O*-acetylhydroshengmanol-3-*O*-β-D-xylopyranoside, (24*S*)	50 000	5600
6	24-epi-24-*O*-acetylhydroshengmanol (Δ-16,17)-enol ether-3-*O*-β-D-xylopyranoside, (24*R*)	>50 000	1000
7	24-*O*-acetylhydroshengmanol-3-*O*-α-L-arabinopyranoside, (24*S*)	>50 000	32 000
8	24-*O*-acetylhydroshengmanol (Δ-16,17)-enol ether-3-*O*-α-L-arabinopyranoside, (24*S*)	16 000	1000
9	24-*O*-acetylhydroshengmanol-3-*O*-β-D-xylopyranoside, (24*R*)	16 000	3000
10	hydroshengmanol (Δ-16,17)-enol ether, (24*S*)	20 000	6300
11	hydroshengmanol (Δ-16,17)-enol ether-3-*O*-β-D-xylopyranoside, (24*S*)	43 000	1200
12	24-*O*-acetylhydroshengmanol (Δ-16,17)-enol ether, (24*S*)	>10 000	>10 000

In modified H4 cells stably expressing wild type human APP,[24] purified **1** had an IC_{50} for the lowering of Aβ42 of 100 nM (Figure 2). This effect on the amount of Aβ42 contrasted with a much lesser effect on the production of Aβ40 with an IC_{50} of 6.3 μM. These highly encouraging results triggered further examination of the effect of **1** on the production of different lengths of Aβ using immunoprecipitation-mass spectrometric (IP-MS) analysis of cell-conditioned media of cells treated with **1**.[34,35] These analyses revealed that 1.0 μM **1** affects Aβ production to selectively shift the average length of the total pool of observable Aβ(1−*x*) to shorter lengths, with Aβ37 and Aβ39 increased relative to Aβ40 while Aβ38 is decreased (Figure 3A, C). This profile is different from that reported for other GSMs in which Aβ42 is reduced, and Aβ38 is increased.[12,13] Compound **1** lowers production of Aβ42 and at the same time the proportions of Aβ37 and Aβ39 relative to the level of Aβ40 are increased. In the CHO cell line 7PA2[36] in which the V717F mutant of APP[37,38] is expressed, resulting in a much greater proportion of Aβ42 as compared to expression of wild-type APP, **1** is also effective (Figure 3B, D) demonstrating efficacy of **1** is not limited to wild-type APP alone. Relative to Aβ40, Aβ42 is lowered while the pattern of increases in Aβ37

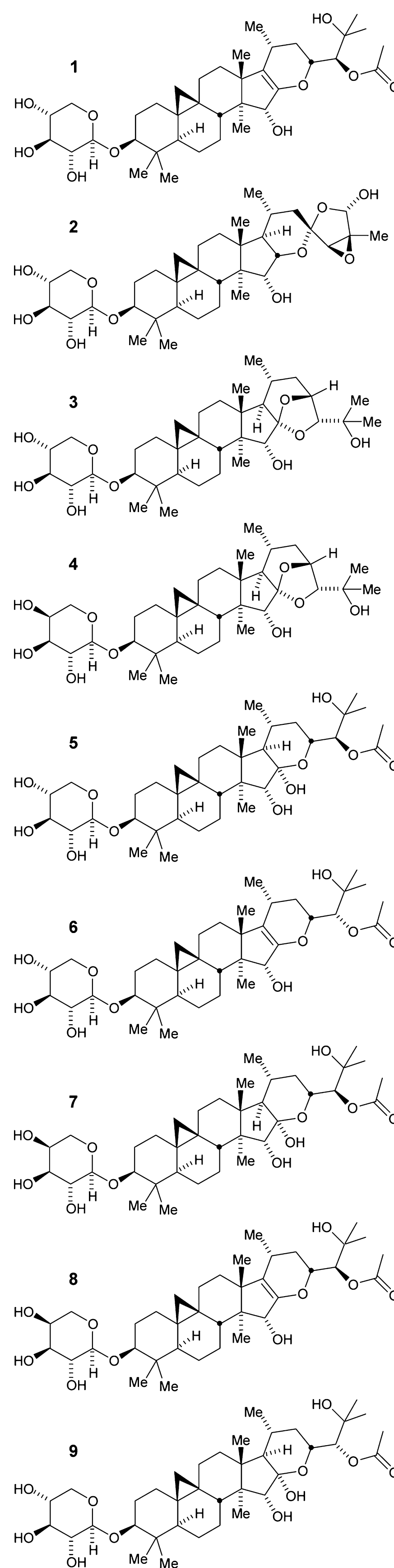


Figure 1. Structures of compounds **1**−**9** isolated from black cohosh extract.

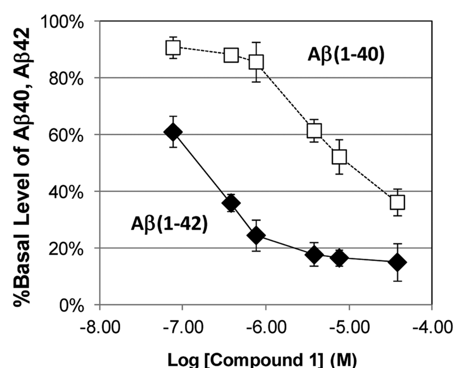


Figure 2. Dose response to treatment of H4 cells with compound **1**: Aβ42 (◆) and Aβ40 (□).

and Aβ39 was maintained. Processing of APP and the distribution of the various lengths of Aβ which are produced from it are dependent on the stepwise efficiency and processivity of γ-Sec-mediated proteolysis.[8,39,40] Changes in the distribution of Aβ lengths effected by **1** suggest that **1** modulates γ-Sec to result in increased processing of longer Aβ peptides to produce Aβ39 and Aβ37. Elucidation of the novel mechanisms of action of this class of GSMs will be disclosed in due course.

Part of the goal in pursuing GSMs is to allow normal activity of γ-Sec on its other substrates, such as Notch. To probe how **1** affected γ-Sec-mediated processing, we examined the production of other fragments of APP as well as the production of NICD in cells treated with **1**. In CHO-7W cells expressing wild-type APP, **1** did not appear to significantly block overall APP processing based on the lack of excess accumulation of the APP fragments C99/C83 (Figure 4A). The γ-Sec inhibitor DAPT, by contrast, causes an increase in these fragments by blocking their further processing to release Aβ. In HEK-N7 cells expressing Notch, **1** did not affect formation of NICD at concentrations of ∼10 μM or below (Figure 4B). Any changes in production of APP fragments or NICD occurred at concentrations of **1** well above that required to significantly and selectively lower the level of Aβ42 production in these cells (Figure 4C). This ability to potently lower Aβ42 production while allowing normal processing of other essential γ-Sec substrates such as Notch suggests that **1** and related compounds are less likely to have the toxicity associated with γ-Sec inhibitors or less selective modulators. Taken together, these data suggest that **1** may bind to the γ-Sec complex or the γ-Sec-APP complex in such a manner as to modulate the cleavage of APP to reduce Aβ42 and cause a change in the distribution of Aβ(1−*x*)s that are produced, consistent with a mode of modulation similar to but distinct from of other small molecule GSMs.

In vivo activity of **1** was probed by dosing mice and assaying for lowering of Aβ42 in brain. Treatment of normal mice with 10 μg (∼0.4 mg/kg) of **1** via intracerebroventricular (i.c.v.) administration resulted in a 15% lowering of Aβ42 (Figure 5). Compound **1** was further characterized to probe its chemical and biological stability. The related C24 alcohol des-acetyl xyloside **10** was readily obtained by mild base treatment of **1**

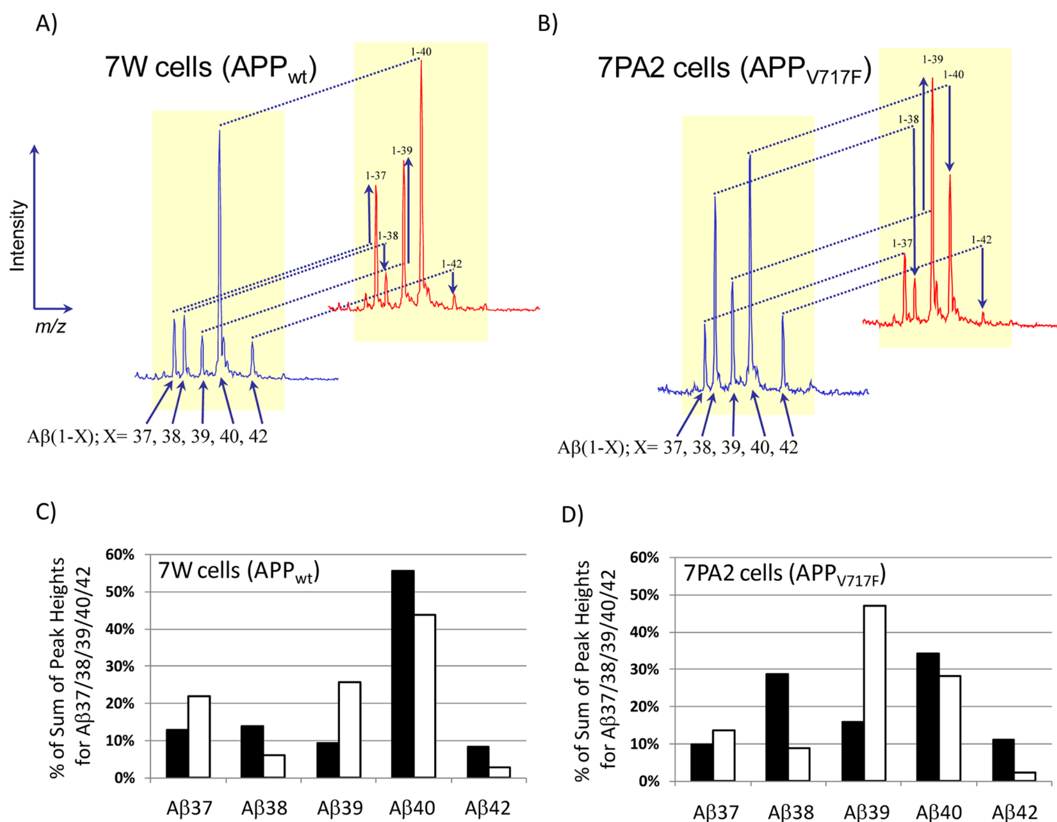


Figure 3. IP-MS analysis of cell-conditioned media of CHO-7W cells (A) and CHO-7PA2 cells (B) treated with 1 μM **1**. Mass spectral data for the mass range encompassing Aβ(1−*x*) for *x* = 37−42 (*m*/*z* ∼4000−4500) are shown for control and treated cells. Ion peak heights were used as measures of the abundance of different lengths of Aβ(1−*x*) and graphed to show relative abundance among the five main species of Aβ(1−*x*) observed in this mass range for CHO-7W (C) and CHO-7PA2 (D) cells: control untreated cells (■), treated cells (□).

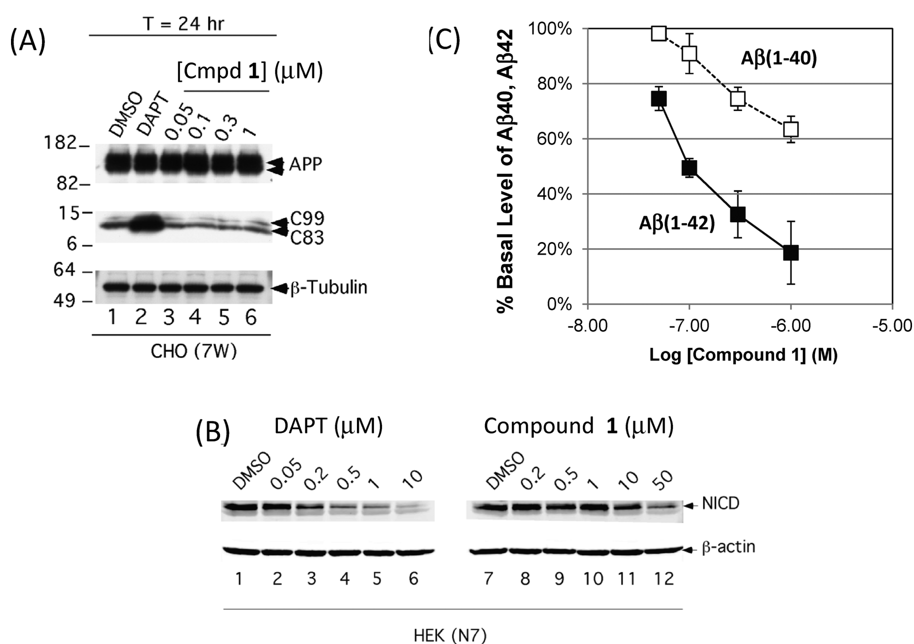


Figure 4. (A) Western blot analysis of APP processing in CHO-7w cells. GSI DAPT (10 μM) inhibits total γ-Sec activity, causing an increase in the accumulation of APP fragments C99/C83. GSM **1** does not cause such an accumulation at up to 1 μM. (B) Western blot analysis of Notch processing in HEK-N7 cells. DAPT, at levels that substantially eliminate total Aβ production, also reduces production of NICD, while levels of **1** that reduce Aβ42 (C) still allow Notch processing to proceed substantially intact.

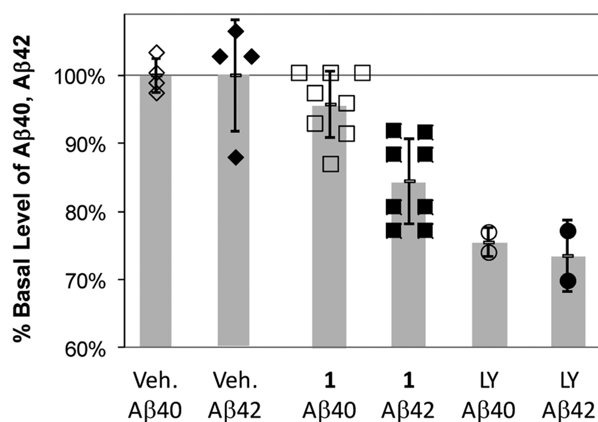


Figure 5. Effects of **1** on Aβ levels in mouse brain. Values for individual mice are indicated by markers. CD-1 normal mice were dosed i.c.v. with **1** (10 μg), LY450139 (positive control), or vehicle control. Levels of Aβ40/42 in mouse brain were compared to values in control brain as 100% for each corresponding ELISA. Vehicle controls: Aβ40 (◇), Aβ42(◆). **1**: Aβ40 (□), Aβ42 (■). GSI LY450139: Aβ40 (○), Aβ42(●).

(Scheme 1). Attempts to remove the xylosyl group from **1** using typical treatment with strong acid were found to result in a complex mixture of degradation products. An enzymic approach,[41] however, was able to remove the carbohydrate moiety under mild conditions and provide the C3 alcohol aglycone product **11**. As with the preparation of **10**, mild base treatment of aglycone **11** was found to readily remove its acetyl group to obtain the tetraol des-acetyl aglycone **12**. All three of these derivatives were significantly less active than **1** to lower levels of Aβ42 (Table 2) but did provide reference standards to probe whether these derivatives of **1** might be formed as metabolites of **1**.

Analysis of plasma and brains of normal mice dosed orally with **1** showed multihour exposure of **1** was maintained but much higher exposure was observed of the anticipated metabolites (Figure 6). A higher level of **10** (5–10-fold), the deacetylated derivative of **1**, was seen relative to the levels of **1** seen at the same time points. The dual metabolite **12** was observed increasing at later time points and was the major species observed in brain at the 8 h time point, as compared to the parent compound **1**.

To further probe the pharmacological selectivity of **1**, a preliminary screen for off-target pharmacology was performed. This panel of 63 targets included assays for receptors for neurotransmitters, steroids, growth factors and hormones, brain and gut peptides, nitric oxide synthase (NOS), as well as selected ion channels (including cardiotoxicity related hERG) and enzymes. Gratifyingly, no binding or modulating activity outside of the normal variability of the assay (±20%) was observed at a concentration of 1 μM, and no effect greater than baseline was observed in this screen at up to 10 μM of **1** except, interestingly, that a moderate effect was observed in the melatonin receptor binding assay.

Cellular toxicity was carefully monitored during all described studies of Aβ42-lowering activity. Both lactate dehydrogenase (LDH) activity and MTS redox dye-based assays are used to monitor cell viability in control and **1**-treated cells (data not shown). Observations are that if an Aβ42-specific reduction is obtained, no changes in cell viability are seen by either indicator. At higher concentrations of **1** (10–50 μM) when Aβ40 is lowered significantly, LDH and MTS assays suggest that cell viability is reduced.

Compound **1** represents a structurally novel class of GSMs that potently and selectively modulates APP processing to lower levels of Aβ42 and reduce its proportion among various species of Aβ leading to a shorter average length of the total Aβ pool. In addition, by virtue of the effects on Aβ37–39, **1** provides a pharmacological profile distinct from other GSMs. These properties fit the profile of a compelling chemical scaffold suitable to identify a GSM of possible use to treat AD.

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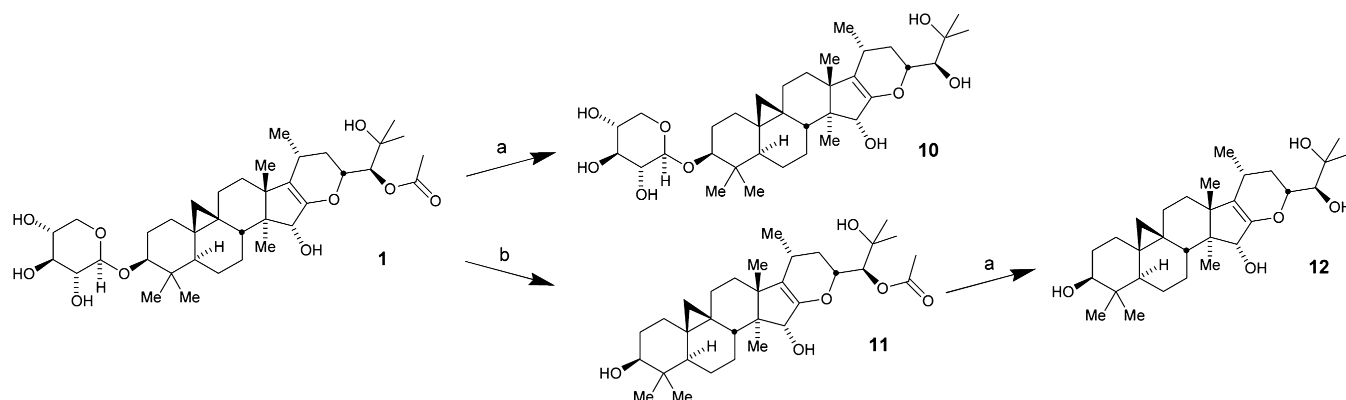
Scheme 1. Conversion of 1 to Des-acetyl Xyloside 10, Aglycone 11, and Des-acetyl Aglycone 12: (a) KOH/MeOH; (b) Cellulase, H_2O-MeOH, pH 6.0



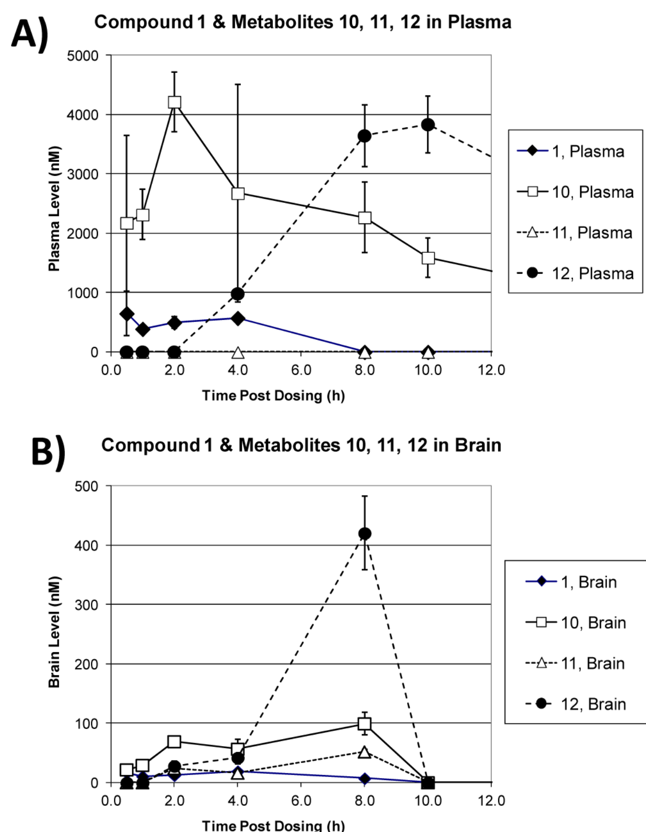


Figure 6. Pharmacokinetic data for **1** and metabolites **10**−**12**. CD-1 normal mice were dosed orally with **1** (30 mg/kg). Plasma and brain were analyzed at 0.5, 1, 2, 4, 8, and 10 h postdosing by LC-MS. Plasma (A) and brain (B) levels of **1** (◆), **10** (□), **11** (△), and **12** (●).

Preliminary structure−activity relationship (SAR) studies based on comparison with other isolated related compounds, and the three derivatives prepared, indicate the novel enol ether structure of **1** in combination with the nearby acetoxy group and polar xylosyl moiety combine to form a novel structure associated with high potency. Not surprisingly, in vitro and in vivo metabolic lability studies with **1** suggests that the specific use of this compound as a potential therapeutic is not practical but that further studies to adapt the structure of **1** to design and synthesize improved compounds are worthy of continued effort.

■ METHODS

Additional detailed data is included in the Supporting Information including a table of IUPAC names and CAS Registry numbers for reported compounds; a scheme of the fractionation protocol and associated chromatographic data; mass spectral data from IP-MS studies; details on synthetic chemistry for the preparation of compounds **10**−**12**; study of the pharmacokinetics and metabolism of **1** in mice; and a protocol for isolation of **1**.

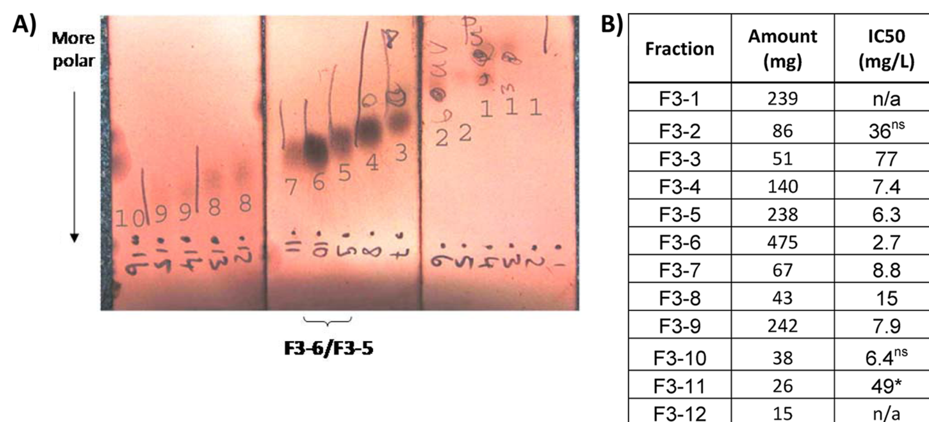
Analytical Instrumentation. NMR spectra were recorded on a Varian INOVA-600 (600 MHz for ^{1}H, 151 MHz for ^{13}C), INOVA-500 (500 MHz for ^{1}H and 125 MHz for ^{13}C), and INOVA-400 (400 MHz for ^{1}H, 100 MHz for ^{13}C) instruments. HPLC-MS was performed using an Agilent 1100 Series HPLC system equipped with a diode array detector and connected to a Quattro II spectrometer (Micromass/Waters). Flash chromatography was performed using a Teledyne ISCO CombiFlash system. HPLC fractionation was performed using an Agilent 1100 Series HPLC system equipped with an Agilent Eclipse XDB-C18 column (9.4 × 250 mm, 5 μm particle diameter) coupled to a Teledyne ISCO Foxy 200 fraction collector.

Black Cohosh Extracts. Semisolid "raw" black cohosh extracts prepared by alcoholic extraction of powdered root and rhizome of *Actaea racemosa* were obtained as gifts from Bio-Botanica (Hauppauge, NY), BI Nutraceuticals (Islandia, NY), and Pure World, Inc. (now Naturex; Hackensack, NJ). All extracts were similar in chromatographic and biological properties. Commercial preparations of extract already diluted into carrier diluents such as alcohols were found to be far less suitable for fractionation. If unable to obtain undiluted raw extract, we suggest the preparation of extract directly from commercially available root powder obtained from a reputable supplier.

Fractionation of Black Cohosh Extract. Raw extract (6.4 g) was dispersed in water, and this mixture was sequentially extracted with EtOAc and *n*-BuOH. The two organic extracts were combined and evaporated to dryness, yielding 3.1 g of brown solid (fraction 2). This material was suspended in MeOH−water (4:1 v/v), and the resulting mixture was mechanically stirred for 30 min to obtain an emulsion which was combined with silica gel (ICN silica 32-63 60 Å) and evaporated under vacuum to a dry powder. This extract−silica mixture was suspended in MeOH−CH_2Cl_2 (1:20 v/v) and added to the top of a silica column previously equilibrated with MeOH−CH_2Cl_2 (1:20 v/v) which was then eluted with a step gradient of MeOH−CH_2Cl_2 (1:20, 1:10, 1:7, 1:5 v/v) to obtain a series of 12 fractions (fraction set 3) which were evaporated to dryness, yielding 1.66 g of total material. The most potent and selective fractions in this group corresponded to the largest mid-R_f fractions F3-5 and F3-6 (Table 3). These fractions were selected for further study.

Upon concentration of fraction F3-5, a white solid (238 mg) was obtained. Following recrystallization and NMR analysis, this material was identified as actein, **2**,[28,29] which was essentially inactive.

Table 3. Fractions of Black Cohosh Extract Obtained Following Normal Phase Chromatography on Silica Gel Eluted with Methylene Chloride−MeOH[a]



Fraction	Amount (mg)	IC50 (mg/L)
F3-1	239	n/a
F3-2	86	36[ns]
F3-3	51	77
F3-4	140	7.4
F3-5	238	6.3
F3-6	475	2.7
F3-7	67	8.8
F3-8	43	15
F3-9	242	7.9
F3-10	38	6.4[ns]
F3-11	26	49*
F3-12	15	n/a

[a](A) Thin-layer chromatographic analysis of isolated fractions. (B) Weights of isolated material from fractions and IC_{50} to lower level of Aβ42. Total amount of isolated fractions as dried solids: 1.66 g. n/a, not active; ns, non-specific; *, less potent but good selectivity for Aβ42. Fractions F3-5 and F3-6 were chosen for further fractionation based on potency.

Partial concentration of fraction F3-6 afforded a crystalline white solid (76 mg) which NMR analysis indicated was an approximately 2:1 mixture of the known cimigenols **3** (cimigenol-3-*O*-beta-D-xylopyranoside)[30] and **4** (cimigenol-3-*O*-alpha-D-arabinopyranoside)[30,31] plus traces of closely related cimigenols. The mother liquors of this crystallization were combined and evaporated to a residue (fraction F3-6-1, 404 mg) that was then subjected to further analysis and fractionation by C-18 HPLC to obtain a series of fractions (F4-1 to F4-7).

Fraction F4-6 was further separated into five fractions (F5-1 to F5-5) by C-18 RP-HPLC, with the most active fraction being F5-4. By NMR, this fraction was predominantly the known 24-*O*-acetylhydroshengmanol-3-*O*-beta-D-xylopyranoside (**5**).[25] Repeated crystallization of F5-4 provided highly purified **5** (fraction F6-R). Further fractionation of the mother liquor of F5-4 by C-8 HPLC (AcCN-H_2O) separated F5-4 into a series of additional fractions F6. The most active of these fractions, F6-6, when analyzed by NMR was found to contain ~90% 24-*O*-acetylhydroshengmanol 3-*O*-beta-D-xylopyranoside (Δ-16,17)-enol ether (**1**). Further separation of this fraction provided pure **1** and a small amount of the C24-epimer of **1**, (24*R*) 24-*O*-acetylhydroshengmanol 3-*O*-beta-D-xylopyranoside (Δ-16,17)-enol ether (**6**).

Fractions F5-2 and F5-5 were further separated by C-8 RP-HPLC. In F5-2 were the L-arabinosyl analogues **7** and **8** of the D-xylosyl shengmanol **5**, and the corresponding enol ether **1**. In F5-5 were additional quantities of the cimigenol glycosides **3** and **4**, along with the C-24 epimer **9** of shengmanol xyloside **5**.

ELISA for Aβ(1−40) and Aβ(1−42). ELISAs to quantify levels of Aβ(1−40) and Aβ(1−42) in CHO-2B7 or H4 cell-conditioned media were performed as previously described.[23,24]

Cell-Based Assay for Aβ(1−42)/Aβ(1−40) Production. This assay was performed essentially as described previously.[23,24] Extract fractions and isolated compounds were dissolved in DMSO at 5−50 mg/mL to make stock solutions for assay. Stock solutions of test samples were then added to assay media in a typical range of 0.0001%−0.5% v/v. For comparison, potency of samples was calculated on a weight basis as the concentration of sample required to lower the level of Aβ42 or Aβ40 by 50%.

APP and Notch Processing. APP and Notch processing were studied in CHO-7W[42,43] and HEK293-N7 cell lines. CHO 7w and HEK N7 cells cultured in DMEM/10% fetal bovine serum were plated in 12-well plates (N7: coated with poly-D-lysine, Sigma) and treated during 24 h with different concentrations of **1** or 10 μM of the γ-secretase inhibitor DAPT[44] (*N*-[(3,5-difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine-1,1-dimethylethyl ester, Sigma). Total protein extracts were prepared in 1% NP-40-HEPES buffer (50 mM HEPES pH 7.0, 150 mM NaCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$) containing protease inhibitor mix (PI, Roche Applied Science), clarified by centrifugation for 1 h at 16 000g, 4 °C, and quantified by the BCA protein assay reagent kit (Pierce). For Western blot analysis, proteins were separated using 4−20% Tris-glycine polyacrylamide gels, transferred to polyvinylidene difluoride, and probed with C9 antiserum (Selkoe laboratory) to detect APP-FL and its APP-CTFs. β-Tubulin was used as a loading control. Immunoreactive bands were detected using the ECL-plus chemiluminescent kit (Amersham Biosciences) and exposed onto Blue Sensitive photographic film (Marsh Bio Products, Inc.).

Production of Aβ40 and Aβ42 in cell conditioned media of CHO-7w cells treated with **1** was measured as follows by ELISA. The conditioned media were collected, PI added, and assayed for Aβ40 and Aβ42 by ELISA as previously described.[45] The capture antibodies were 2G3 (to Aβ residues 33−40) for the Aβ40 species and 21F12 (to Aβ residues 33−42) for the Aβ42 species.

HEK293 cells expressing mouse Notch1-Δ*E* (N7 cells)[46] cultured in DMEM/10% fetal bovine serum/1% penicillin-streptomycin (Invitrogen) serum were plated in 12-well plates coated with poly-D-lysine (Sigma) and treated during 24 h with the indicated concentrations of **1** or the γ-secretase inhibitor DAPT. Total protein extracts were prepared in 1% NP-40-HEPES buffer (50 mM HEPES pH 7.0, 150 mM NaCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, PI), clarified by centrifugation for 1 h at 16 000g, 4 °C, and quantified by the BCA protein assay reagent kit (Pierce). For Western blot analysis, proteins were run on 4−12% Bis-Tris gels and transferred onto PVDF membranes to detect NICD with the Notch-specific Ab1744 antibody (1:1000; Cell Signaling Technology, Beverly, MA), which is selective for the free N-terminus of NICD. The fluorescence signal was detected with the Odyssey infrared imaging (LICOR, Bad Homburg, Germany) scanner. β-Actin was used as a loading control.

Cellular Toxicity. Toxicity of isolated compounds including **1** was assessed using both lactate dehydrogenase (LDH) activity and MTS redox dye-based assays to monitor cell viability in control and experimental cell samples

IP-MS Analysis of Cell-Conditioned Medium. CHO-7w and CHO-7PA2 cells cultured in DMEM/10% fetal bovine serum were plated in 10 cm dishes and treated during 24 h with DMSO as vehicle control, **1** (0.1, 1 μM), the γ-secretase inhibitor DAPT (20 μM), and sulindac sulfide (100 μM) (see Supporting Information). The conditioned media were harvested and frozen for subsequent analysis. IP-MS analysis for Aβ(1−*x*) was performed as previously described.[34,35]

Side Effect Pharmacology Screening Panel. Compound **1** was tested in the standard general side effect PROFILE panel number 1 at Caliper Life Sciences (Hanover, MD) at 1 and 10 μM. In addition to

the 63 standard assay targets, assays for COX1 and COX2 were included in the panel as well.

Treatment of CD-1 Mice via icv Dosing of 1. Normal Female CD-1 mice were dosed i.c.v. with **1** (10 μg). Levels in brain of Aβ(1−40) and Aβ(1−42) were quantified by ELISA and are reported as percent of vehicle control treated animals. As a positive control, orally dosed GSI LY450139 was used at 10 mg/kg.[47]

Metabolism Studies. Normal CD-1 male mice (Charles River Laboratories, Portage, MI) were dosed orally with 30 mg/kg **1** (3.75 mg/mL solution in 2% sodium dodecyl sulfate) at Ricerca Biosciences (Concord, OH). At varying time points following dosing, mice were sacrificed, blood and brain collected, and plasma isolated and frozen for analysis. RP-HPLC coupled with tandem mass spectrometry was used to analyze for the presence of **1** and anticipated metabolites **10**−**12** at Tandem Laboratories (Woburn, MA). Authentic samples of each compound were used to establish analytical methods and quantitative standard curves.

■ ASSOCIATED CONTENT

Supporting Information

Additional detailed data: NMR data for compound **1**; a table of IUPAC names and CAS Registry numbers for reported compounds; a scheme of the fractionation protocol and associated chromatographic data; mass spectral data from IP-MS studies; and details on synthetic chemistry for the preparation of compounds **10**−**12**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

F.S. performed botanical extract fractionation and compound identification. T.D.M, D.Y., and P.C.F. performed biological assays. MAF sourced botanical extracts. T.D.M., S.P.C., W.F.A., and M.A.F. performed chemistry. R.W. performed IP-MS analysis of conditioned cell culture medium. M.A.F., C.B.E., J.C., and D.S. contributed to experimental plans and provided guidance.

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Notes

The authors note the following relevant financial interests: M.A.F., F.S., S.P.C., and W.F.A. are named as inventors on one or more patents and patent applications related to compounds discussed in this paper. C.B.E. is an inventor on a patent related to this paper. M.A.F., T.D.M., W.F.A., C.B.E., and D.S. either hold equity and/or options on equity in Satori Pharmaceuticals. No other authors have relevant competing interests.

■ ABBREVIATIONS

Aβ, amyloid-beta peptide; APP, amyloid precursor protein; CHO, Chinese hamster ovary; C-8, octyl; C-18, octadecyl; COX, cyclooxygenase; DAPT, (*N*-[(3,5-difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine-1,1-dimethylethyl ester; ELISA, enzyme-linked immunosorbent assay; GSI, γ-secretase inhibitor; GSM, γ-secretase modulator; HEK, human embryonic kidney; RP-HPLC, reverse-phase high-performance (high-pressure) liquid chromatography; i.c.v., intracerebroventricular; IP-MS, immunoprecipitation-mass spectrometry; LC-MS, liquid chromatography−mass spectrometry; NSAID, nonsteroidal anti-inflammatory drug; SAR, structure−activity relationship(s)

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