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The Natural Products Beauveriolide I and III: a New Class of β-Amyloid-Lowering Compounds

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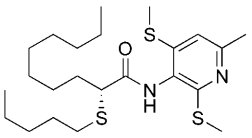
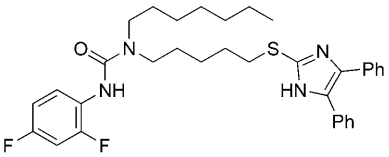
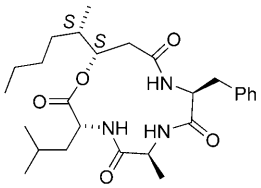
Alzheimer's disease (AD) is now the most common form of dementia, affecting up to 15 million people worldwide. A common pathogenic event that occurs in all forms of AD is the progressive accumulation of amyloid-β peptide (Aβ) in brain loci responsible for cognition.[1] Genetic and epidemiological data support a role for altered cholesterol homeostasis in the pathogenesis of AD,[2] with polymorphisms in a number of cholesterol-related genes being linked to elevated central levels of Aβ.[3] Recent studies suggest that intracellular levels of cholesteryl esters (CEs) correlate closely with Aβ production and secretion,[4–6] and that the cellular enzyme responsible for CE production, acyl-CoA cholesterol acyltransferase (ACAT), might play a central role in the regulation of Aβ processing. Kovacs and co-workers[5,6] have recently shown that the ACAT inhibitors CP-113,818 and DuP-128 decrease Aβ secretion in Chinese hamster ovary (CHO) cells stably transfected with human amyloid precursor protein (APP_{751}). Dorsal implantation



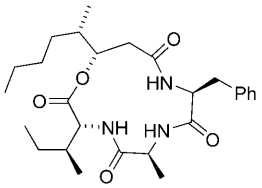
CP-113,818



DuP-128



beauveriolide I (**1**)



beauveriolide III (**2**)

of CP-113,818 in a transgenic murine model of familial AD resulted in the reduction of brain Aβ plaque load.[4]

The depsipeptide natural products beauveriolide I (**1**) and beauveriolide III (**2**) were originally isolated from the fungal strain *Beauveria* sp. FO-6979 during a screening program for inhibitors of lipid droplet accumulation in murine macrophages.[7] The assignment of the absolute stereochemistry of the 3-hydroxy-4-methyloctanoic acid (HMA) as 3*S*,4*S* and the first formal syntheses of all stereoisomers of **1** and **2** were reported in 2006.[8] Depsipeptides **1** and **2** prevent lipid droplet accumulation in primary mouse peritoneal macrophages through the inhibition of ACAT and importantly, they are orally active and reduce atherogenic lesions in mouse models of atherosclerosis.[9] These depsipeptides are currently being studied as a new approach for the treatment of atherosclerosis.[9,10]

Given our ongoing interest in the pathogenesis of atherosclerosis[11] and AD,[12] and our expertise in synthetic approaches to cyclodepsipeptides[13] we wondered whether **1** and **2** may be a valid chemical scaffold that would, for the first time, link orally active ACAT inhibition to therapeutically relevant neuronal Aβ reduction. Towards this goal, we report herein a new flexible homochiral synthesis of the fungal depsipeptide natural products beauveriolides I and III, and reveal that these cyclodepsipeptides are potent inhibitors of cellular $Aβ_{40}$ and $Aβ_{42}$ secretion in vitro.

Our synthesis of **1** and **2** commences with a *cis*-crotylation of PMB-protected aldehyde **3** that proceeds with high diastereoselectivity (>98:2) and enantioselectivity (>95:5) to give *cis*-alcohol **4** (Scheme 1). Homologation of **4** proceeds by initial TBS protection of the secondary alcohol, hydroboration of the terminal double bond, TPAP-mediated oxidation of the resultant primary alcohol, and Wittig olefination to give the TBS-protected *cis*-alkene **5a** (>85% *Z* as determined by ^{1}H NMR analysis).

A key aspect in the synthesis of depsipeptides is the strategy for macrocycle formation, either lactonization or lactamization. Our synthesis, which prepares the key (3*S*,4*S*)-HMA in a masked form (compound **5a**), is amenable to both approaches. However, for brevity only the macrolactamization approach is described in Scheme 1. Thus, the TBS group of **5a** is removed, and the secondary alcohol **5b** is esterified with either *N*-Boc-D-Leu-OH (for **1**), or *N*-Boc-D-*allo*-Ile-OH (for **2**) with $Sc(OTf)_3$ catalysis: conditions that result in no measurable racemization of the amino acid esters **6a** or **6b**.

Completion of the syntheses of **1** and **2** are then entirely parallel and involve PMB removal, primary alcohol oxidation, coupling of H_2N-L-Phe-L-Ala-CO-*t*Bu, alkene reduction, and protecting group removal to give amino acids **7a** or **7b**. The critical macrolactamization reaction was carried out with HATU/HOAt activation and proceeded in >70% yield with both **7a** and **7b** to give **1** and **2**, respectively.

With synthetic **1** and **2** in hand, we studied the ability of these depsipeptides to decrease cholesterol esterification in the stable transgenic CHO cell line (7WD10) expressing human

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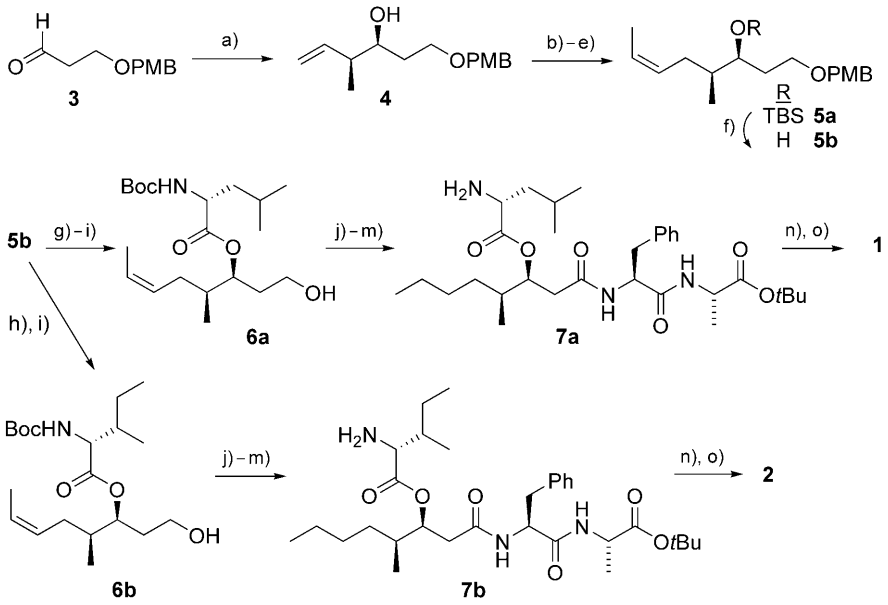

Scheme 1. Synthesis of **1** and **2**: a) *cis*-2-butene, *t*BuOK, *n*BuLi, −78 °C→−50 °C, (+)-B-methoxydiisopinocamphorylborane, $BF_3{\cdot}OEt_2$, 78%; b) TBSOTf, 2,6-lutidine, 95%; c) 9-BBN, THF, H_2O_2, NaOH, 82%; d) TPAP, NMO, 96%; e) $EtPPh_3$, NaHMDS, 83%; f) TBAF, 97%; g) DIC (2.4 equiv), DMAP (2.0 equiv), *N*-Boc-D-Leu (2.0 equiv), $Sc(OTf)_3$ (0.4 equiv), 87%; h) DIC (2.4 equiv), DMAP (2.0 equiv), *N*-Boc-D-*allo*-Ile (2.0 equiv), $Sc(OTf)_3$ (0.4 equiv), 79%; i) DDQ, CH_2Cl_2/H_2O; j) Dess–Martin, CH_2Cl_2; k) $NaClO_2$, NaH_2PO_4, 2-methyl-2-butene, *t*BuOH/H_2O; l) EDC, HOBt, H_2N-L-Phe-L-Ala-COO-*t*Bu, CH_2Cl_2, 55% over four steps: i–l; m) H_2, Pd/C (5%), EtOAc (quant); n) TFA/CH_2Cl_2; o) HATU, HOAt, collidine, DMF; **1**: 74%, **2**: 71%. 9-BBN: 9-borabicyclo[3.3.1]nonane; DDQ: 2,3-dichloro-5,6-dicyano-1,4-benzoquinone; DIC: 1,3-diisopropylcarbodiimide; DMAP: 4-dimethylaminopyridine; DMF: *N*,*N*-dimethylformamide; EDC: *N*′-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide; HATU: *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HMDS: 1,1,1,3,3,3-hexamethyldisilazane; HOAt: 1-hydroxyazobenzotriazole; HOBt: 1-hydroxybenzotriazole; NMO: *N*-methylmorpholine-*N*-oxide; PMB: *p*-methoxybenzyl; TBAF: tetra-*n*-butylammonium fluoride; TBS: *tert*-butyldimethylsilyl; Tf: trifluoromethanesulfonyl; TPAP: tetra-*n*-propylammonium perruthenate.

APP_{751}.[14] Cultured 7WD10 cells incubated with depsipeptide **1** or **2** (5 μM) in media and cosolvent (DMSO, 0.1%, *v*/*v*) exhibit lower cellular CE levels within 12 h. This decrease in CE reaches a maximum level (~5% CEs relative to vehicle-treated cells) after 24–48 h incubation with **1** and **2** (data not shown). Depsipeptides **1** and **2** exhibit a dose-dependent decrease in CE levels in 7WD10 cells, with IC_{50} values of 0.08 ± 0.02 and 0.17 ± 0.08 μM, respectively after 96 h incubation (Figure 1A). These values are similar to those of **1** and **2** in primary murine macrophages.[9] Importantly, the cellular total cholesterol (TC), that is, the sum of free cholesterol (FC) and CE, remains essentially unchanged in the CHO cells throughout incubation with **1** and **2** (data not shown). This null effect of the beauveriolides on TC causes a shift in cellular cholesterol from esterified to nonesterified and is in agreement with what has been observed for other ACAT inhibitors, such as CP-113,818 and DuP-128, in other cell lines.[6] This shift supports the notion that the decrease in CE levels by **1** and **2** is due to ACAT inhibition alone and not a result of either reduced endogenous cholesterol biosynthesis through the inhibition of HMG-CoA reductase, or reduced uptake of cholesterol from media. This lowering of cellular CEs is expressed macroscopically as a decrease in the number of cytoplasmic lipid droplets. Using fluorescence microscopy and cellular lipid droplet staining with Oil Red O dye, we observe this macroscopic effect as an almost complete lack of lipid droplets in **1**- and **2**-treated 7WD10 cells relative to control cells (Figures 1B–D).

We next investigated whether beauveriolide treatment has any impact on Aβ secretion from this cell line using an ELISA-based method that can quantify both

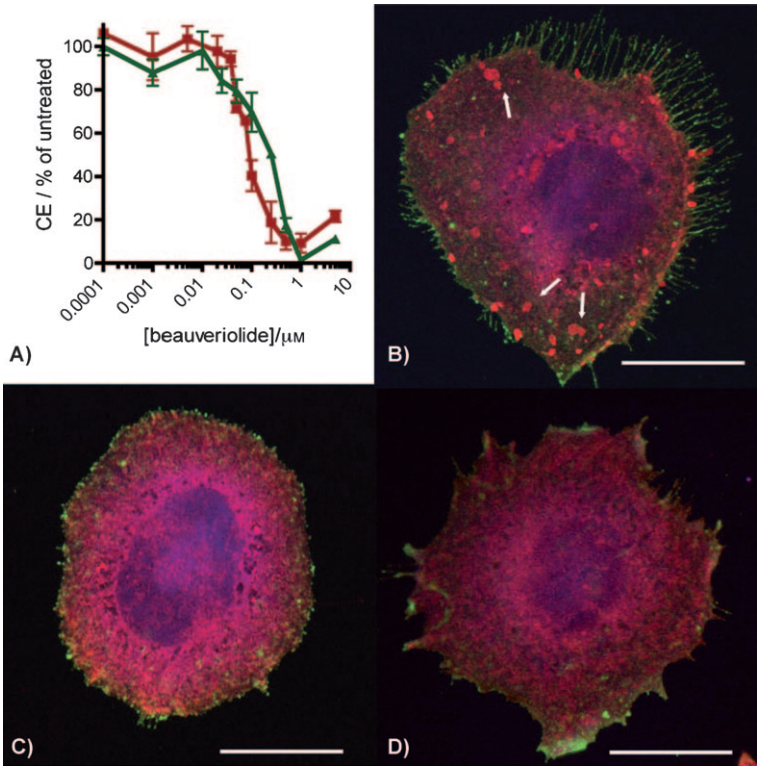

Figure 1. Depsipeptides **1** and **2** decrease cholesteryl ester (CE) levels and fat droplets in the stable transgenic CHO cell line 7WD10 expressing human APP_{751}. A) Cellular CE levels as a percentage of vehicle-treated cells after incubation with **1** (■) or **2** (▲) for 96 h; values are plotted as the mean ± SEM of at least three experiments. B)–D) Fluorescence microscopy of fixed live 7WD10 CHO cells stained with Oil Red O dye, which stains lipid droplets red (indicated with white arrows), and lysenin, which stains membrane sphingomyelin (green), after 48 h culture with: B) vehicle (DMSO 0.1%, *v*/*v*, in media), C) **1** (1 μM), or D) **2** (1 μM); scale bar = 10 μm.

$A\beta_{40}$ and $A\beta_{42}$ (Figure 2). Incubation of 7WD10 cells with **1** and **2** (1 μM) for up to four days causes a decrease in $A\beta_{40}$ and $A\beta_{42}$ secretion, the intensity and time profile of which is compound dependent (Figure 2A). The time dependence of the decrease in Aβ secretion induced by **1** (~25 and 10% reduction in $A\beta_{40}$ and $A\beta_{42}$ levels after four days, respectively) reaches a maximum effect after one to two days, paralleling this depsipeptide's effect on lowering CE levels (see above). Beauveriolide III (**2**) is more effective at lowering Aβ secretion (~45 and 65% reduction in $A\beta_{40}$ and $A\beta_{42}$ levels after four days, respectively) than **1**, and the time dependence of compound **2** in decreasing Aβ levels does not parallel the CE effect as closely as **1**, with $A\beta_{40}$ and $A\beta_{42}$ levels still decreasing after incubation for four days (Figure 2A).

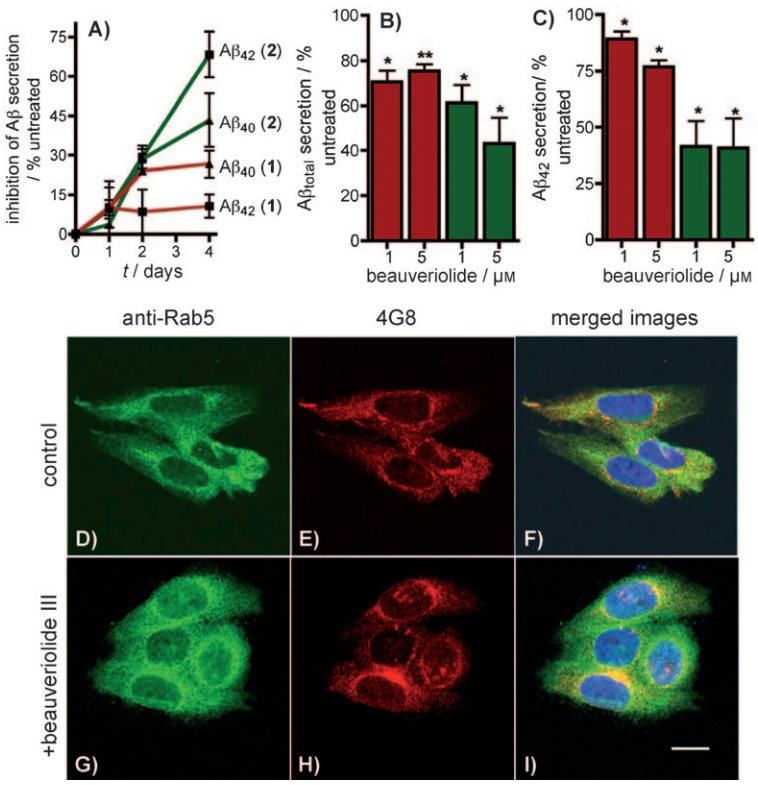

Figure 2. Depsipeptides **1** and **2** decrease Aβ secretion from the CHO cell line 7WD10, and do not perturb trafficking of Aβ. A) Time-dependent changes in $A\beta_{40}$ (▲) and $A\beta_{42}$ (■) secretion by 7WD10 cells incubated with either **1** (——) or **2** (——) at 1 μM as determined by ELISA analysis of $A\beta_{40}$ and $A\beta_{42}$ in conditioned media standardized to total protein. B) Concentration dependence of **1** (red bars) and **2** (green bars) on $A\beta_{total}$ secretion from the 7WD10 cell line. C) Concentration dependence of **1** (red bars) and **2** (green bars) on $A\beta_{42}$ secretion from the 7WD10 cell line. Data in A)–C) are expressed as the mean ±SEM of at least three separate experiments and are recorded as a percent of vehicle-treated cells (0.1% DMSO, *v/v*). Statistical analysis was performed by using a student *t* test; significance: $^*p < 0.05$ and $^{**}p < 0.01$. Lower panels show confocal microscopy images of 7WD10 cells incubated with D)–F) vehicle (0.1% DMSO, *v/v*), or G)–I) beauveriolide III (1 μM) for 48 h. Cells were fixed with paraformaldehyde and immunolabeled with either a rabbit anti-Rab5 antibody with an Alexa-488-labeled (green) secondary antibody (D, G), or the murine anti-Aβ antibody 4G8, with an Alexa-543-labeled (red) secondary antibody (E, H). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Images were recorded with a Zeiss LSM510 confocal microscope at 63×, and image analysis and merges were performed with LSM image examiner software (v4.2). Note: yellow color in the merged images (F, I) corresponds to colocalized Rab5 and Aβ; scale bar = 10 μm.

The time-dependent effects of the beauveriolides **1** and **2** on Aβ secretion were next validated in a concentration-effect study (Figures 2B and C). Significant reductions in $A\beta_{total}$ ($A\beta_{40} + A\beta_{42}$) levels relative to vehicle-treated cells (DMSO, 0.1%, *v/v*) were observed after incubation for 96 h with either **1** or **2** at 1 and 5 μM. The most effective compound at decreasing Aβ secretion is depsipeptide **2**, which induces a mean decrease in $A\beta_{total}$ of 39±10% ($^*p < 0.05$) at 1 μM and 57±14% ($^*p < 0.05$) at 5 μM (Figure 2B). Depsipeptide **2** also had a more profound effect on decreasing $A\beta_{42}$ secretion than **1**, with reductions of 58±12% ($^*p < 0.05$) and 59±14% ($^*p < 0.05$), respectively (Figure 2C).

To assess whether the decrease in Aβ secretion induced by compound **2** corresponds to measurable changes in intracellular Aβ localization, immunolabeling was performed with the anti-Aβ antibody 4G8 and an anti-Rab5 antibody (Figures 3D–I). This microscopy study revealed that beauveriolide III treatment does not alter the known early endosomal localization and trafficking of Aβ, as merged images of vehicle-treated (DMSO, 0.1%, *v/v*) and depsipeptide **2**-treated cells show the same degree of colocalization of Aβ and Rab5 (the early endosome-localized Rab GTPase;[15] Figures 2F and I).

The nature of the link between ACAT inhibition, CE homeostasis, and the biogenesis of Aβ is still an ongoing area for study and has been linked to either intracellular Aβ accumulation and mistrafficking[16] or a change in the enzymatic processing of APP.[17] Our microscopy studies of 7WD10 cells treated with the ACAT inhibitor beauveriolide III reveal no indication of either altered localization or accumulation of Aβ (Figure 2I) and no mistrafficking of normal lipids such as sphingomyelin (Figure 1D). These observations are in line with a mechanism of decreased biogenesis of Aβ recently proposed by Kovacs and co-workers[17] that involves enzymatic N-terminal processing of APP and APP degradation dependent on cellular CE levels.

In conclusion, we have developed a flexible homochiral synthesis of the fungal-derived beauveriolide natural products, and have shown them to be extremely effective at lowering cellular Aβ secretion in the transgenic CHO cell line 7WD10 that expresses human APP_{751}. What makes this study particularly significant is the potency and rapid onset with which these depsipeptides, especially beauveriolide III (**2**), decrease the cellular secretion of $A\beta_{42}$. While the major peptide generated by APP processing is $A\beta_{40}$, the more hydrophobic $A\beta_{42}$ is the predominant peptide found in senile AD plaques, and therefore reducing central levels of this peptide is seen as a clearly unmet therapeutic need. Incubation with compound **2** at 1 μM decreases $A\beta_{42}$ secretion by ~58% after four days and ~30% after two days relative to vehicle-treated cells. Previous studies with the ACAT inhibitor CP-113,818 on cellular $A\beta_{42}$ secretion (using a CHO cell line expressing human APP_{751}) revealed no

measurable decrease in $A\beta_{42}$ secretion after incubation for four days at a concentration of 1 mM. In fact, a tenfold greater concentration of CP-113,818 (10 μM) was required to induce the same decrease in $A\beta_{42}$ secretion as depsipeptide **2**.

As mentioned above, current ACAT inhibitors such as CP-113,818 and DuP-128 are limited by low bioavailability. They are absorbed poorly from the gut and are rapidly metabolized in blood or tissue. This is the case for the lipidic pyridylamide CP-113,818, for which an in vivo study to assess Aβ in mice involved a 60-day release pellet formulation that was implanted under the skin.[4] In contrast, beauveriolide III has shown oral bioavailability in both apoE knockout and LDL receptor knockout mouse models of atherosclerosis.[9] Therefore, if our discovery that these compounds are highly effective at decreasing Aβ secretion in vitro can be translated to an in vivo setting, then coupled with the known pharmacokinetic properties of the beauveriolides, it may well be demonstrated that these depsipeptides are excellent new candidates for reducing the senile plaque burden in AD.

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Keywords: Alzheimer's disease · amyloid beta-peptides · cholesterol · inhibitors · natural products

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