

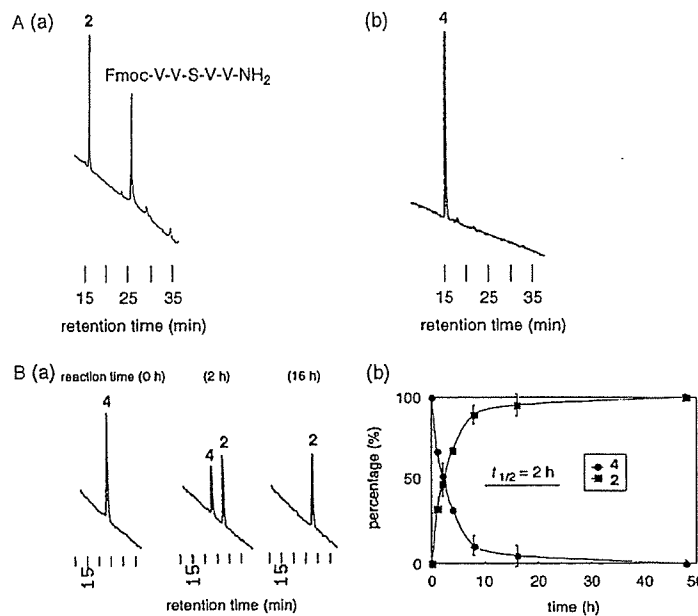
**Figure 1** (A) 'O-Acyl isopeptide method': the synthetic strategy for difficult sequence-containing peptides via the O-N intramolecular acyl migration reaction of O-acyl isopeptide. (B) application of the O-acyl isopeptide method for the synthesis of pentapeptide **1**, and (C) application of the O-acyl isopeptide method for the synthesis of pentapeptide **2**.

[7,8]. The O-N intramolecular acyl migration is a well-known reaction in Ser/Thr-containing peptides [9-11]. The O-acyl isopeptide method required no special building blocks and markedly improved the synthetic yields of difficult sequence-containing pentapeptides such as Ac-Val-Val-Pns-Val-Val-NH<sub>2</sub> (**1**, Pns: phenylnorstatine, (2*R*,3*S*)-3-amino-2-hydroxy-4-phenylbutanoic acid [12-16], Figure 1B) and Ac-Val-Val-Ser-Val-Val-NH<sub>2</sub> (**2**, Figure 1C) [7,8]. For example, in the synthesis of **2** based on this method, O-acyl isopeptide **4** was synthesized as a major product with high purity (Figure 2A-b), while a large amount of undesired Fmoc-containing peptide was obtained in a conventional Fmoc-based solid-phase method (Figure 2A-a). This indicated that the branched ester structure improved coupling and deblocking efficacy during SPPS, by suppressing the unfavourable nature of difficult sequence-containing peptides originating from secondary structure formation among the peptide chains on the resin. In addition, O-acyl isopeptides with a newly formed amino group attained reasonable H<sub>2</sub>O- and MeOH-solubility required in HPLC purification by the formation of salt. Furthermore, from recent research of water-soluble prodrugs [17-24] and O-acyl isopeptides [7,8] based on O-N intramolecular acyl migration, it has been established that the purified O-acyl isoform can quantitatively be converted to the

original N-acyl form in a short time with no side reaction at pH 7.4 (Figure 2B). These results suggest that the 'O-acyl isopeptide method' is advantageous for synthesizing small difficult sequence-containing peptides. This study therefore focused on one of the larger difficult sequence-containing peptides, amyloid  $\beta$  peptide (A $\beta$ ) 1-42.

Amyloid  $\beta$  peptides (A $\beta$ s) are the main proteinaceous component of amyloid plaques found in the brains of Alzheimer's disease (AD) patients [25]. Neuritic plaques, pathognomonic features of AD, contain abundant fibrils formed from A $\beta$ , which has been found to be neurotoxic *in vivo* and *in vitro* [26]. The predominant forms of A $\beta$  mainly consist of 40- and 42-residue peptides (designated A $\beta$ 1-40 and A $\beta$ 1-42, respectively), which are proteolytically produced from amyloid precursor protein (APP) by enzymatic reactions [27]. Since A $\beta$ 1-42 is thought to play a more critical role in amyloid formation and the pathogenesis of AD than A $\beta$ 1-40, many studies using synthetic A $\beta$ 1-42 have been carried out to clarify the involvement of A $\beta$ 1-42 in AD [28-32].

However, A $\beta$ 1-42 is defined as a difficult sequence-containing peptide with a high hydrophobicity and forms aggregates in various media [33-44]. In particular, due to its low solubility and broad elution under acidic or neutral conditions, the conventional



**Figure 2** A: HPLC profiles of (a) crude peptide **2** and (b) its *O*-acyl isopeptide **4** [0–100% CH<sub>3</sub>CN for 40 min, 230 nm]. B: (a) periodical HPLC profiles (0–100% CH<sub>3</sub>CN for 40 min, 230 nm) and (b) the graph for conversion of *O*-acyl isopeptide **4** to **2** via the *O*-*N* intramolecular acyl migration in phosphate buffered saline (pH 7.4, 25 °C).

HPLC purification of synthesized A $\beta$ 1–42 in the aqueous TFA–acetonitrile system is too laborious to remove impurities accumulated during solid-phase peptide synthesis (SPPS). To improve the synthetic difficulty of this peptide, strong acylation reagents such as HATU [36–38,41] and Fmoc-amino acid fluorides [37], the DBU/DMF system for effective Fmoc removal [39], and sulfoxide protection for Met<sup>35</sup> to suppress aggregation [44] were employed. For effective HPLC purification, this peptide has also been purified under basic conditions [36,41]. Moreover, A $\beta$ 1–42 synthesis has been achieved by the segment condensation of fully protected peptide fragments in a solution method employing chloroform–phenol mixed solvent [40].

From the aspect of biological experiments, A $\beta$ 1–42 is also problematic due to its large extent of aggregation in a standard storage solution such as dimethylsulfoxide (DMSO) [45]. In addition, since this peptide undergoes time- and concentration-dependent aggregation in the acetonitrile–water used for HPLC purification [35], the dry, the purified peptide adopts different structures and aggregation states [46]. However, numerous studies have established that neurotoxicity and the kinetics of aggregation are directly related to the assembly state in solution. Thus, depending on the commercial source, peptide batch, and the aggregation condition, considerable discrepancies might exist in the biological data across different laboratories as well as within the same laboratory over time. Generally, to disaggregate the A $\beta$ 1–42, the peptide needs to be predissolved in dilute base solution. Therefore, an 'in situ' system that

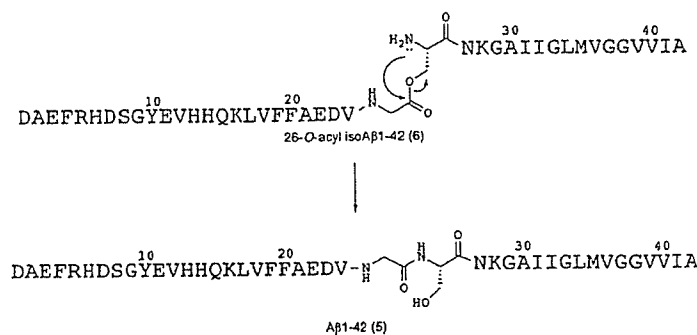
could prepare intact monomer A $\beta$ 1–42 in a soluble form under physiological conditions would be a powerful tool in understanding its inherent pathological function. To create such a system, (1) a novel propeptide possessing high solubility and no aggregate character during HPLC purification and long-term storage as a solution and (2) the capability of intact A $\beta$ 1–42 production under physiological conditions would be desired.

Based on this background, the idea was conceived that the '*O*-acyl isopeptide method' could be applied to the synthesis of A $\beta$ 1–42 via a novel water-soluble isopeptide of A $\beta$ 1–42, i.e. '26-*O*-acyl isoA $\beta$ 1–42 (26-ALA $\beta$ 42, **6**)' (Figure 3) [47,48]. This overcomes the problems in the synthesis and storage of A $\beta$ 1–42. Although there are two Ser residues in A $\beta$ 1–42 at positions 8 and 26 with the capability of *O*-*N* intramolecular acyl migration, Ser<sup>26</sup> was selected for *O*-acylation, since the adjacent Gly<sup>25</sup> does not epimerize during ester bond formation (Figure 3). A previous rapid communication [47] reported the efficient synthesis of A $\beta$ 1–42 by the '*O*-acyl isopeptide method'. This paper describes the detailed synthesis of A $\beta$ 1–42, the importance of resin selection and the analysis of side reactions in the *O*-acyl isopeptide method.

## MATERIALS AND METHODS

### General

All protected amino acids and resins were purchased from Calbiochem-Novabiochem Japan Ltd (Tokyo). Other chemicals



**Figure 3** 'O-Acyl isopeptide method' for the synthesis of A $\beta$ 1-42 (5): The production of A $\beta$ 1-42 (5) via the O-N intramolecular acyl migration of 26-O-acyl isoA $\beta$ 1-42 (6).

were mainly purchased from commercial suppliers, Wako Pure Chemical Ind. Ltd (Osaka, Japan), Nacalai Tesque (Kyoto, Japan), Aldrich Chemical Co. Inc. (Milwaukee, WI) and Peptide Institute, Inc. (Osaka, Japan) and were used without further purification. MALDI-TOF MASS spectra were recorded on Voyager DE-RP using  $\alpha$ -cyano-4-hydroxy cinnamic acid as a matrix. FAB-MS was performed on a Jeol JMS-SX102A spectrometer equipped with the JMA-DA7000 data system. Analytical HPLC was performed using a C18 reverse phase column (4.6  $\times$  150 mm; YMC Pack ODS AM302) with a binary solvent system: a linear gradient of CH<sub>3</sub>CN in 0.1% aqueous TFA at a flow rate of 0.9 ml min<sup>-1</sup> (temperature: 40°C), detected at 230 nm. Preparative HPLC was carried out on a C18 reverse phase column (20  $\times$  250 mm; YMC Pack ODS SH343-5) with a binary solvent system: a linear gradient of CH<sub>3</sub>CN in 0.1% aqueous TFA at a flow rate of 5.0 ml min<sup>-1</sup> (temperature: 40°C), detected at 230 nm. Solvents used for HPLC were of HPLC grade.

### Solid-Phase Peptide Synthesis

The Fmoc-amino acid side-chain protections were selected as follows: tBu (Asp, Glu, Ser, Thr, Tyr), Boc (Lys), Pmc (Arg), Trt (Asn, Gln, His). Generally, the peptide chains were assembled by the sequential coupling of activated N $\alpha$ -Fmoc-amino acid (2.5 eq) in DMF (1.5–2 ml) in the presence of 1,3-diisopropylcarbodiimide (DIPCDI, 2.5 eq) and 1-hydroxybenzotriazole (HOBT, 2.5 eq) with a reaction time of 2 h at room temperature. The resins were then washed with DMF ( $\times$ 5) and the completeness of each coupling was verified by the Kaiser test. N $\alpha$ -Fmoc deprotection was carried out by treatment with piperidine (20% v/v in DMF) (2 ml, 1 min  $\times$ 1 and 20 min  $\times$ 1), followed by washing with DMF (1.5 ml,  $\times$ 10) and chloroform (1.5 ml,  $\times$ 5). If necessary, the coupling and deprotection cycles were repeated. After the peptide-resins were washed with methanol (1.5 ml,  $\times$ 5) and dried for at least 2 h *in vacuo*, the peptides were cleaved from the resin with TFA in the presence of thioanisole, *m*-cresol and distilled water (92.5:2.5:2.5:2.5) for 90 min at room temperature, concentrated *in vacuo*, and precipitated with diethyl ether (4–8 ml) at 0°C followed by centrifugation at 3000 rpm for 5 min ( $\times$ 3). The resultant peptides were dissolved or suspended with water and lyophilized for at least 12 h. The crude products were purified by preparative reversed-phase HPLC with 0.1% aqueous TFA-CH<sub>3</sub>CN system as an eluant, immediately frozen at -78°C, and lyophilized

at least 12 h. Purified peptides were stored dry at -20°C until use.

**Ac-Val-Val-Pns-Val-Val-NH<sub>2</sub> (1, by the conventional method).** The peptide **1** was synthesized on Rink amide aminomethyl (AM) resin (200 mg, 0.148 mmol) according to the general Fmoc-based solid-phase procedure described. After the resin was washed with DMF (1.5 ml,  $\times$ 5), Fmoc-Val-OH (125.6 mg, 0.37 mmol) and Fmoc-Pns-OH (185.4 mg, 0.44 mmol) were coupled in the presence of DIPCDI (57.9  $\mu$ l, 0.37 mmol) and HOBT (56.7 mg, 0.37 mmol) in DMF (1.5 ml) for 2 h according to the sequence. The Fmoc-group was removed by 20% piperidine/DMF. N-Acetylation was carried out with acetic anhydride (20.9  $\mu$ l, 0.222 mmol) in the presence of TEA (20.7  $\mu$ l, 0.148 mmol) for 2 h. The peptide was cleaved from the resin using TFA (5 ml) in the presence of thioanisole (136.3  $\mu$ l), *m*-cresol (136.3  $\mu$ l) and distilled water (136.3  $\mu$ l) for 90 min at room temperature, concentrated *in vacuo*, washed with diethyl ether, centrifuged, suspended with water and lyophilized to give the crude peptide (64.6 mg). Subsequently, this crude peptide (20 mg) was saturated in DMSO (1–2 ml), filtered using a 0.46  $\mu$ m filter unit, and immediately injected into preparative HPLC with a 0.1% aqueous TFA-CH<sub>3</sub>CN system. The peak fractions were collected and immediately lyophilized to afford the desired peptide **1** as a white amorphous powder. Yield: 2.0 mg (6.9%); HRMS (FAB): calcd. for C<sub>32</sub>H<sub>53</sub>N<sub>6</sub>O<sub>7</sub>(M + H)<sup>+</sup>: 633.3976, found: 633.3982; HPLC analysis at 230 nm: purity was higher than 98%.

**Ac-Val-Val-Pns-Val-Val-NH<sub>2</sub> (1, by the O-acyl isopeptide method).** After preparation of the H-Val-Val-NH-resin (Rink amide AM resin, 200 mg, 0.126 mmol) in the same manner described in the synthesis of **1** using the conventional method, Boc-Pns-OH (111.6 mg, 0.378 mmol) was coupled in the presence of DIPCDI (59.2  $\mu$ l, 0.378 mmol) and HOBT (57.9 mg, 0.378 mmol) in DMF (1.5 ml). Subsequent coupling with Fmoc-Val-OH (128.3 mg, 0.378 mmol) to the  $\alpha$ -hydroxy group of Pns was performed using the DIPCDI (59.2  $\mu$ l, 0.378 mmol)-DMAP (3.1 mg, 0.0252 mmol) method in CH<sub>2</sub>Cl<sub>2</sub> (1.5 ml) for 16 h ( $\times$ 2), followed by the coupling of another Val residue, N-acetylation using Ac<sub>2</sub>O (14.3  $\mu$ l, 0.15 mmol)-TEA (17.5  $\mu$ l, 0.126 mmol), TFA (4.4 ml)-thioanisole (117.2  $\mu$ l)-*m*-cresol (117.2  $\mu$ l)-distilled water (117.2  $\mu$ l) treatment for 90 min at room temperature, concentration *in vacuo*, diethyl ether wash, centrifugation, suspension with water and lyophilization to give the crude O-acyl isopeptide **3** (51.3 mg).

Subsequently, this crude peptide (20 mg) was dissolved in methanol (500  $\mu$ l), filtered using a 0.46  $\mu$ m filter unit, and immediately injected into preparative HPLC with a 0.1% aqueous TFA-CH<sub>3</sub>CN system. The peak fractions were collected and immediately lyophilized, affording the desired *O*-acyl isopeptide **3** as a white amorphous powder (18.7 mg, 58.3%). HRMS (FAB): calcd for C<sub>32</sub>H<sub>53</sub>N<sub>6</sub>O<sub>7</sub>(M + H)<sup>+</sup>: 633.3976, found: 633.3979; HPLC analysis at 230 nm: purity was higher than 99%.

Purified *O*-acyl isopeptide **3** was then dissolved in phosphate-buffered saline (PBS, pH 7.4) at room temperature (1 mg ml<sup>-1</sup>) and stirred for 5 min at room temperature. The resultant precipitate was centrifuged and washed with water and methanol followed by drying *in vacuo* to give **1** as a white powder. Yield: 18.5 mg (57.6%); HRMS (FAB): calcd. for C<sub>32</sub>H<sub>53</sub>N<sub>6</sub>O<sub>7</sub>(M + H)<sup>+</sup>: 633.3976, found: 633.3983; HPLC analysis at 230 nm: purity was higher than 99%.

**Ac-Val-Val-Ser-Val-Val-NH<sub>2</sub> (2, by the conventional method).** Peptide **2** was synthesized from Rink amide AM resin (200 mg, 0.126 mmol) and Fmoc-Ser-OH (120.8 mg, 0.315 mmol) in a similar manner to that described for peptide **1** in the conventional method. Yield: 4.1 mg (6.0%); HRMS (FAB): calcd. for C<sub>25</sub>H<sub>47</sub>N<sub>6</sub>O<sub>7</sub>(M + H)<sup>+</sup>: 543.3506, found: 543.3510; HPLC analysis at 230 nm: purity was higher than 95%.

**Ac-Val-Val-Ser-Val-Val-NH<sub>2</sub> (2, by the *O*-acyl isopeptide method).** Peptide **2** was synthesized from Rink amide AM resin (200 mg, 0.126 mmol) and Boc-Ser-OH (65.2 mg, 0.315 mmol) in a similar manner to that described for peptide **1** in the *O*-acyl isopeptide method. *O*-Acyl isopeptide **4**: Yield: 27.7 mg (40.6%); HRMS (FAB): calcd. for C<sub>25</sub>H<sub>47</sub>N<sub>6</sub>O<sub>7</sub>(M + H)<sup>+</sup>: 543.3506, found: 543.3509; HPLC analysis at 230 nm: purity was higher than 99%. **2**: Yield: 27.7 mg (40.6%); HRMS (FAB): calcd. for C<sub>32</sub>H<sub>53</sub>N<sub>6</sub>O<sub>7</sub>(M + H)<sup>+</sup>: 543.3506, found: 543.3499; HPLC analysis at 230 nm: purity was higher than 96%.

**Amyloid  $\beta$  peptide A $\beta$ 1-42 (5, by the conventional method).** The chlorotriyl chloride resin (200 mg, 0.3 mmol) and Fmoc-Ala-OH (49.4 mg, 0.15 mmol) were taken to the manual solid-phase reactor under an argon atmosphere and stirred for 2.5 h in the presence of DIPEA (26.2  $\mu$ l, 0.15 mmol) in 1,2-dichloroethane (1.5 ml). After washing with DMF (1.5 ml,  $\times$ 5), capping was performed with MeOH (200  $\mu$ l) in the presence of DIPEA (52.5  $\mu$ l, 0.3 mmol) in DMF for 20 min. After washing with DMF ( $\times$ 5), DMF-H<sub>2</sub>O (1:1,  $\times$ 5), CHCl<sub>3</sub> ( $\times$ 2) and MeOH ( $\times$ 2) followed by drying *in vacuo*, the loading ratio was determined (0.1 mmol) photometrically from the amount of Fmoc chromophore liberated upon treatment with 50% piperidine/DMF for 30 min at 37°C. The sequential Fmoc-protected amino acids (0.25 mmol) were manually coupled in the presence of DIPCDI (36.4  $\mu$ l, 0.25 mmol) and HOBt (35.6 mg, 0.25 mmol) for 2 h in DMF (1.5 ml) after the removal of each Fmoc group by 20% piperidine-DMF for 20 min (resin: 465.4 mg). The resulting protected peptide-resin (128.9 mg) was treated with TFA (2.5 ml)-*m*-cresol (64.5  $\mu$ l)-thioanisole (64.5  $\mu$ l)-H<sub>2</sub>O (64.5  $\mu$ l) for 90 min, concentrated *in vacuo*, washed with diethyl ether, centrifuged, suspended with water and lyophilized to give the crude peptide **5** (53.6 mg). This peptide (20 mg) was dissolved in TFA (2 ml)-H<sub>2</sub>O (1 ml) in the presence of NH<sub>4</sub>I (12 mg, 0.08 mmol)

and dimethylsulfide (6  $\mu$ l, 0.08 mmol) and stood for 60 min at 0°C. After concentration *in vacuo*, the crude peptide was dissolved in hexafluoroisopropanol, filtered using a 0.46  $\mu$ m filter unit, applied to preparative HPLC, and eluted using 0.1% aqueous TFA-CH<sub>3</sub>CN. The peak fractions were collected and immediately lyophilized to afford the desired peptide **5** as a white amorphous powder. Yield: 3.6 mg (7.2%); MALDI-MS (TOF): M<sub>calc</sub>: 4514.04; M + H<sub>found</sub>: 4515.48; HPLC analysis at 230 nm: purity was >94%; the retention time on HPLC (0-100% CH<sub>3</sub>CN for 40 min, 230 nm) of synthesized **5** was identical to that of commercially available A $\beta$ 1-42 (Peptide Institute, Inc., Osaka, Japan).

**Amyloid  $\beta$  peptide A $\beta$ 1-42 (5, in *O*-acyl isopeptide method).** After protected A $\beta$ 27-42-resin (chlorotriyl chloride resin, 0.1 mmol) was synthesized in the same manner as described in the synthesis of A $\beta$ 1-42 in the conventional method, Boc-Ser-OH (48.1 mg, 0.25 mmol) was coupled by the DIPCDI (36.4  $\mu$ l, 0.25 mmol)-HOBt (35.6 mg, 0.25 mmol) method for 2 h in DMF (1.5 ml). Coupling with Fmoc-Gly-OH (82.9 mg, 0.3 mmol) was performed using the DIPCDI (43.7  $\mu$ l, 0.3 mmol)-DMAP (2.3 mg, 0.02 mmol) method in CH<sub>2</sub>Cl<sub>2</sub> (1.5 ml) for 16 h ( $\times$ 2). Subsequent amino acid residues were coupled after the removal of each Fmoc group using 20% piperidine for 20 min (resin: 536.9 mg). Resulting protected peptide-resin (176.5 mg) was treated with TFA (3.4 ml)-*m*-cresol (88.3  $\mu$ l)-thioanisole (88.3  $\mu$ l)-H<sub>2</sub>O (88.3  $\mu$ l) for 90 min, concentrated *in vacuo*, washed with diethyl ether, centrifuged, suspended with water and lyophilized to give the crude *O*-acyl isopeptide **6** (97.3 mg). This peptide **6** (40 mg) was dissolved in TFA (4 ml)-H<sub>2</sub>O (2 ml) in the presence of NH<sub>4</sub>I (21.8 mg, 0.17 mmol) and dimethylsulfide (11  $\mu$ l, 0.17 mmol) and stood for 60 min at 0°C. After concentration *in vacuo*, the crude peptide was dissolved in hexafluoroisopropanol, filtered using a 0.46  $\mu$ m filter unit, applied to preparative HPLC, and eluted using a 0.1% aqueous TFA-CH<sub>3</sub>CN. The desired fractions were collected and immediately lyophilized to afford peptide **6** as a white amorphous powder. Yield: 22.9 mg (33.6%); MALDI-MS (TOF): M<sub>calc</sub>: 4514.04; M + H<sub>found</sub>: 4515.26; HPLC analysis at 230 nm: purity was >96%.

The purified **6** was dissolved in H<sub>2</sub>O and stirred for 48 h at room temperature followed by lyophilization to yield **5** quantitatively as a TFA salt. MALDI-MS (TOF): M<sub>calc</sub>: 4514.04; M + H<sub>found</sub>: 4515.48; HPLC analysis at 230 nm: purity was >95%; the retention time on HPLC (0-100% CH<sub>3</sub>CN for 40 min, 230 nm) of synthesized **5** was identical to that of commercially available A $\beta$ 1-42 (Peptide Institute, Inc., Osaka, Japan).

**26-O-Ser-A $\beta$ 26-42 (15).** After protected A $\beta$ 26-42-resin (**9**, chlorotriyl chloride resin, 0.046 mmol) was synthesized in the same manner as described in the synthesis of **6**, Fmoc-Ser(*t*Bu)-OH (52.9 mg, 0.138 mmol) was coupled using DIPCDI (25.7  $\mu$ l, 0.138 mmol) and DMAP (1.3 mg, 0.0092 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (1.5 ml) for 16 h ( $\times$ 2). The peptide-resin was treated with 20% piperidine-DMF for 20 min and TFA (1.79 ml)-*m*-cresol (45.4  $\mu$ l)-thioanisole (45.4  $\mu$ l)-H<sub>2</sub>O (45.4  $\mu$ l) for 90 min, followed by concentration *in vacuo*, diethyl ether washing, centrifugation, suspension with water and lyophilization to give the crude peptide **15** (26.1 mg). This peptide **15** (10 mg) was dissolved in TFA (1 ml)-H<sub>2</sub>O (0.5 ml) in the presence of NH<sub>4</sub>I (5.45 mg, 0.043 mmol) and dimethylsulfide (11  $\mu$ l, 0.043 mmol) and stood for 60 min at 0°C. After concentration,

the crude peptide was dissolved in DMSO, filtered using a 0.46  $\mu\text{m}$  filter unit, applied to preparative HPLC, and eluted using 0.1% aqueous TFA-CH<sub>3</sub>CN. The desired fractions were collected and immediately lyophilized to afford the titled peptide **15** as a white amorphous powder. MALDI-MS (TOF):  $M_{\text{calc}}$ : 1686.03;  $M + H_{\text{found}}$ : 1687.04.

#### Water-, Methanol- and DMSO-Solubility

Peptides **1–6** were saturated in distilled water, methanol or DMSO and shaken at room temperature. The saturated solutions were passed through a centrifugal filter (0.23  $\mu\text{m}$  or 0.46  $\mu\text{m}$  filter unit, Ultrafree<sup>®</sup>-MC, Millipore). The filtrate was analysed by RP-HPLC to determine the solubility.

#### Stability of *O*-Acyl Isopeptides **2** and **4** in Phosphate Buffered Saline (PBS, pH 7.4)

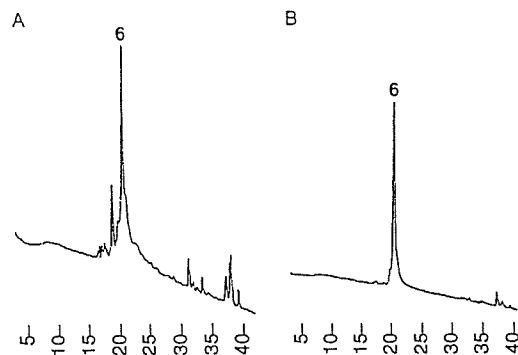
To 495  $\mu\text{l}$  of PBS (pH 7.4) were added 4  $\mu\text{l}$  of DMSO and 1  $\mu\text{l}$  of a solution including **2** or **4** (10 mM in DMSO), and the mixture was stirred at room temperature. At the desired time points, 500  $\mu\text{l}$  of DMSO was added to the samples and 500  $\mu\text{l}$  of the mixture was directly analysed by RP-HPLC. HPLC was performed using a C18 (4.6  $\times$  150 mm; YMC Pack ODS AM302) reverse-phase column with a binary solvent system: linear gradient of CH<sub>3</sub>CN (40%–100%, 30 min) in 0.1% aqueous TFA at a flow rate of 0.9 ml min<sup>-1</sup>, detected at UV 230 nm.

#### Stability of *O*-Acyl IsoA $\beta$ 1–42 (**6**) in Buffers

The conversion profiles at various pHs of **6** were determined in buffered saline in a manner similar to that described for the stability studies of **2** and **4**. To 494–497  $\mu\text{l}$  of buffered saline (pH 7.4: PBS, pH 4.9: PBS, pH 3.5: acetate buffer) was added 3–6  $\mu\text{l}$  of solution including **6** (1 mM in DMSO), and the mixture was incubated at 25 °C or 37 °C in a water bath. At the desired time points 500  $\mu\text{l}$  of hexafluoroisopropanol was added to the samples and 500  $\mu\text{l}$  of the mixture was directly analysed by RP-HPLC.

## RESULTS AND DISCUSSION

In the synthesis of 26-AIA $\beta$ 42 (**6**) based on the *O*-acyl isopeptide method (Scheme 1), Fmoc-Ala-*O*-chlorotriptyl resin (**7**) was employed and Fmoc-protected amino acids were sequentially coupled using the DIPCDI-HOBt method (2 h) after the removal of each Fmoc group with 20% piperidine-DMF (20 min) to give peptide-resin **8**. After Boc-Ser-OH was introduced to **8** by the DIPCDI-HOBt method (2 h), the obtained **9** was coupled with Fmoc-Gly-OH at the  $\beta$ -hydroxy group of Ser using the DIPCDI-DMAP method in CH<sub>2</sub>Cl<sub>2</sub> to obtain ester **10**. 26-AIA $\beta$ 42-resin (**13**) was obtained through the further coupling of additional amino acid residues using the conventional manner. Finally, 26-AIA $\beta$ 42 (**6**) was obtained as a major product (Figure 4A) by treatment with TFA-*m*-cresol-thioanisole-H<sub>2</sub>O (92.5:2.5:2.5:2.5) for 90 min



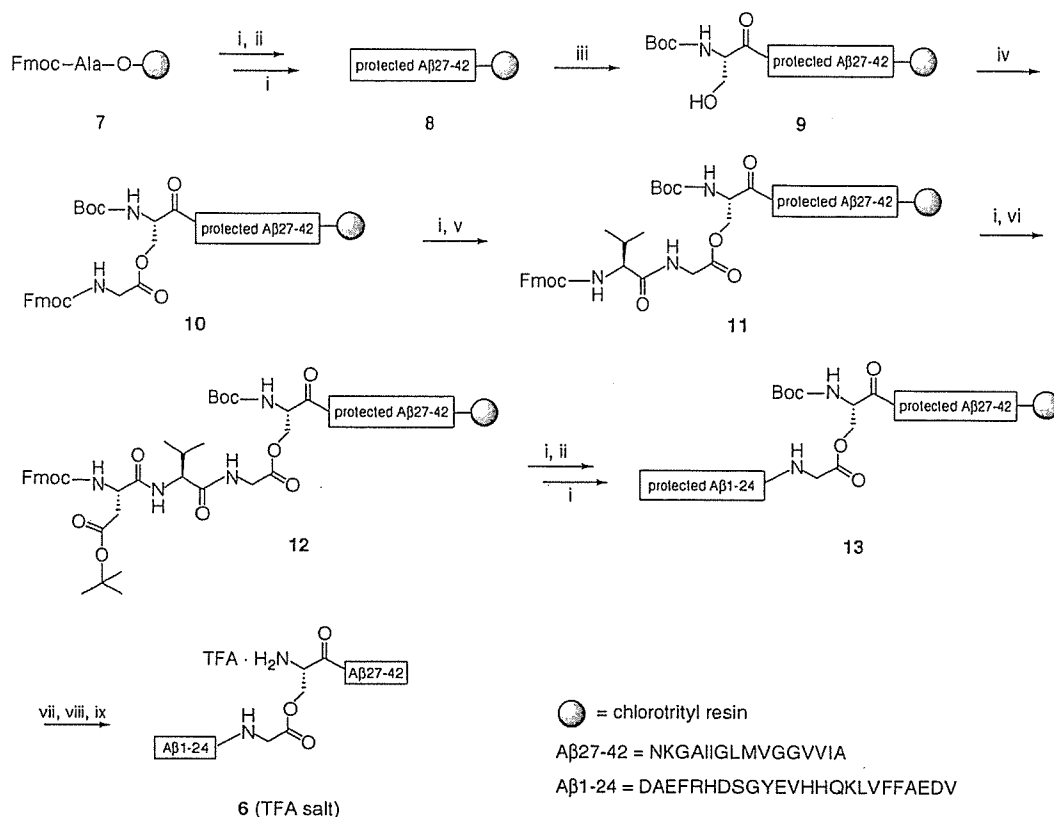
**Figure 4** HPLC profiles of (A) crude and (B) purified 26-AIA $\beta$ 42 (**6**). Analytical HPLC was performed using a C18 reverse-phase column (4.6  $\times$  150 mm; YMC Pack ODS AM302) with a binary solvent system: the linear gradient of CH<sub>3</sub>CN (0–100% CH<sub>3</sub>CN for 40 min) in 0.1% aqueous TFA at a flow rate of 0.9 ml min<sup>-1</sup> (temperature: 40 °C), detected at 230 nm.

followed by reduction with NH<sub>4</sub>I-dimethylsulfide for 60 min in TFA:H<sub>2</sub>O (2:1).

To examine whether the coupling of Boc-Ser-OH with peptide-resin **8** (Scheme 1, step iii) gives a by-product (**14**, Figure 5A) which has an additional insertion of Boc-Ser-OH onto the  $\beta$ -hydroxy group of Ser<sup>26</sup>, an expected by-product **15**, which is a deprotected-cleaved product of **14**, was independently prepared (see Materials and Methods), and compared with the sample deprotected from peptide-resin **9** with a TFA-thioanisole system. However, no by-product **15** was detected in this sample by HPLC analysis, indicating that this side reaction with the formation of **14** can be neglected in the coupling step with Boc-Ser-OH based on the DIPCDI-HOBt method in DMF for 2 h (Figure 5A).

It was confirmed that the esterification of the  $\beta$ -hydroxy group of Ser<sup>26</sup> in **9** with Fmoc-Gly-OH completed on the solid support, since the major product deprotected from peptide-resin **10** by TFA corresponds to *O*-acyl isoA $\beta$ 25–42 by HPLC analysis (data not shown) [TOF-MS:  $M_{\text{calc}}$ : 1878.24;  $M + H_{\text{found}}$ : 1879.29], and A $\beta$ 26–42, which corresponds to the unreacted component, was not detected. In addition, another possible by-product **16** with Fmoc-Gly-Gly sequence caused by elimination of the Fmoc group of **10** by DMAP during the coupling reaction was not detected in the TOF-MS analysis of a deprotected sample of peptide-resin **10** (Figure 5B).

In the HPLC analysis of crude **6** (Figure 4A), A $\beta$ 1–25 (DAEFRHDSGYEVHHQKLVFFAEDVG) was not detected as a by-product, although a very small amount (1.6%, HPLC yield) of A $\beta$ 26–42 (SNKGAIIGLMVGGVVIA) was observed. This indicates that the formed ester bond between Gly and Ser was almost stable in both 20% piperidine and TFA treatment. However, this slight A $\beta$ 26–42 formation in crude **6** might be



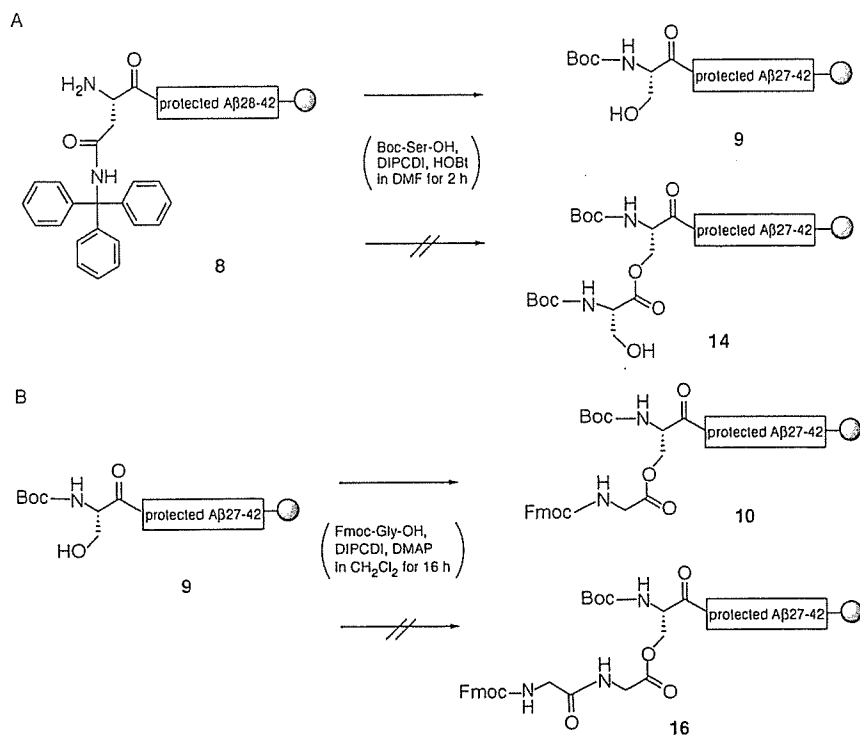
**Scheme 1** Reagents/conditions: (i) 20% piperidine/DMF, 20 min; (ii) Fmoc-AA-OH (2.5 eq), DIPCPI (2.5 eq), HOBT (2.5 eq), DMF, 2 h; (iii) Boc-Ser-OH (2.5 eq), DIPCPI (2.5 eq), HOBT (2.5 eq), DMF, 2 h; (iv) Fmoc-Gly-OH (3.0 eq), DIPCPI (3.0 eq), DMAP (0.2 eq), CH<sub>2</sub>Cl<sub>2</sub>, 16 h × 2; (v) Fmoc-Val-OH (2.5 eq), DIPCPI (2.5 eq), HOBT (2.5 eq), DMF, 2 h; (vi) Fmoc-Asp(OtBu)-OH (2.5 eq), DIPCPI (2.5 eq), HOBT (2.5 eq), DMF, 2 h; (vii) TFA-*m*-cresol-*l*-thioanisole-H<sub>2</sub>O (92.5:2.5:2.5:2.5), 90 min; (viii) NH<sub>4</sub>I (20 eq), dimethylsulfide (20 eq), TFA:H<sub>2</sub>O (2:1), 60 min, 0 °C; (ix) preparative HPLC (the linear gradient of CH<sub>3</sub>CN in 0.1% aqueous TFA).

attributed to the production of **9** by the formation of diketopiperazine during 20% piperidine treatment (20 min) of peptide-resin **11** (Scheme 1). This finding does not correspond to the case of Ac-Val-Val-Ser-Val-Val-NH<sub>2</sub> (**2**) [8] since diketopiperazine formation did not occur in a similar elongation of the peptide chain from the primary hydroxy group of Ser in **4**, suggesting that the less steric-hindered Gly<sup>25</sup> in **11** promoted the cyclization reaction. Therefore, if there are plural Ser or Thr residues in the difficult sequence, the position for converting isopeptide structure is better selected based on the structure of two amino acids residues at the *N*-terminal side next to the isopeptide site (Ser/Thr) to minimize diketopiperazine formation.

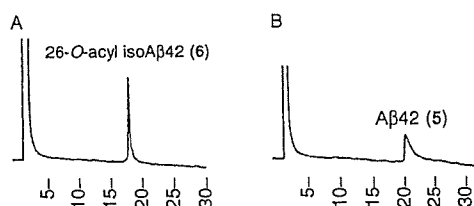
The crude *O*-acyl isopeptide **6** was dissolved in hexafluoroisopropanol, applied to preparative HPLC and eluted using 0.1% aqueous TFA-CH<sub>3</sub>CN. Since **6** was eluted as a narrow single peak (Figures 4 and 6A), it was easily purified using preparative scale HPLC to give pure **6** (Figures 4B and 6A) as a TFA salt with a total isolated yield of 33.6%, calculated from the original loading onto the chlorotrityl resin. This yield was higher

than that obtained in the synthesis of **5** by standard Fmoc-based SPPS (7.2%). Since **5** was eluted as a broad peak (Figure 6B) in HPLC analysis and purification, it was difficult to evaluate the purity and laborious to isolate **5** from impurities as reported [33–44]. In addition, in the synthesis of **6**, no conversion to **5** was observed.

As a resin selection in the synthetic condition setting, 0.16 mmol/g of preloaded Fmoc-Ala-NovaSyn<sup>®</sup>TGA-resin consisting of Tentagel [49] and the TFA-labile linker [50] was also employed (0.0288 mmol) with a similar synthetic procedure as described in the use of chlorotrityl resin for the synthesis of 26-AIAβ42 (**6**), since this type of resin has been well used for the synthesis of Aβ1–42 due to its high swelling characteristic [37–39,41,43]. However, in the use of TGA resin, a large amount of undesired Aβ1–25 and Aβ26–42 (20%–30%, HPLC yield, respectively) was detected on analytical HPLC and MALDI-MS (TOF) (Aβ1–25:  $M_{\text{calc}}$ : 2833.11 M + H<sub>found</sub>: 2934.11, Aβ26–42,  $M_{\text{calc}}$ : 1598.95; M + H<sub>found</sub>: 1599.28) in the resultant crude **6**, indicating that



**Figure 5** Examination whether a side reaction occurred in the reaction of (A) Boc-Ser-OH insertion to **8** (Scheme 1, step iii) and (B) esterification of **9** (Scheme 1, step iv).



**Figure 6** HPLC profiles of pure (A) 26-AIA $\beta$ 42 (**6**) and (B) A $\beta$ 1–42 (**5**). The same quantity (2.3 nmol) of both peptides was applied to the HPLC column as DMSO solution. Analytical HPLC was performed using a C18 reverse-phase column (4.6  $\times$  150 mm; YMC Pack ODS AM302) with a binary solvent system: the linear gradient of CH<sub>3</sub>CN (25%–55% CH<sub>3</sub>CN for 60 min) in 0.1% aqueous TFA at a flow rate of 0.9 ml min<sup>-1</sup> (temperature: 40 °C), detected at 230 nm.

the ester bond between Gly<sup>25</sup> and Ser<sup>26</sup> was significantly cleaved by the treatment of TFA-*m*-cresol-thioanisole-H<sub>2</sub>O (92.5:2.5:2.5:2.5) in the final deprotection. Significant ester bond cleavage occurred in the use of TGA resin, but not more acid-sensitive chlorotrityl resin in the same TFA-cocktail condition suggests that undesired ester bond cleavage mainly occurred in the resin-bound *O*-acyl isopeptides, although the precise reason remains unclear. Consequently, extremely acid-labile 2-chlorotrityl chloride resin is more efficient in the synthesis of *O*-acyl

isopeptides to avoid acid-mediated ester bond cleavage. In the case of Wang resin (0.8 mmol/g), peptide chain elongation was difficult in the 7 to 8 residues from the C-terminal.

The water solubility of **6** (TFA salt) was 15 mg ml<sup>-1</sup>, 100-fold higher than that of A $\beta$ 1–42 (**5**, 0.14 mg ml<sup>-1</sup>). Interestingly, as a slight modification of the peptide chain by inserting one ester bond drastically increased the solubility of the insoluble original peptide with 42 residues, this suggests that *O*-acyl isopeptides totally destroy the secondary structures responsible for the insolubility of the original peptide. In addition, the HPLC analysis of **6** exhibited quite a sharp peak even in the slow gradient system (25%–55% CH<sub>3</sub>CN, 60 min, Figure 6A), while **5** was eluted as a broad peak in the same elution condition (Figure 6B) as reported [33–44]. These results also support that the high assembly characteristic of A $\beta$ 1–42 was suppressed by only one insertion of the isopeptide structure. Recent solution-state NMR studies of the A $\beta$ 1–40 and A $\beta$ 1–42 by Zagorski *et al.* [32] indicated that the Ser<sup>26</sup> residue comprises turn- or bendlike structures that bring two  $\beta$ -sheets in contact and hydrogen bonding among peptide chains, which is associated with  $\beta$ -aggregation. As it was demonstrated that *O*-acyl isopeptide could suppress the unfavourable nature of difficult sequence-containing pentapeptides in the previous study [7,8], this result in the synthesis of **6**

indicates that this method is a powerful strategy for increasing the solubility of even larger peptides. Recent reports by Carpino *et al.* [51] and Mutter *et al.* [52] have also supported our hypothesis that *O*-acyl isopeptide structures possess attractive solubilizing efficacy.

As shown in Figure 7A, purified **6** was quantitatively converted to A $\beta$ 1–42 (**5**) at room temperature in PBS (pH 7.4) with a half-life of 2.6 min and with no side reaction such as hydrolysis of the ester bond. This faster migration may be attributed to the less steric hindrance of the Gly<sup>25</sup> residue. In addition, in PBS (pH 7.4) at 37 °C, this migration was very rapid with a half-life of approximately 1 min and with no side reaction, and migration was completed after 30 min. On the other hand, the TFA salt of **6** was stable at 4 °C for at least 1 month in either a solid state or a DMSO solution. Moreover, as shown in Figure 7B, slower migration was observed at pH 4.9 (PBS) with a half-life of 3 h and no migration at pH 3.5 (acetate buffer) after incubation for 3 h at room temperature. This rapid migration under physiological conditions enables the production of an intact monomer A $\beta$ 1–42, *in situ* to investigate the inherent biological function of A $\beta$ 1–42 in AD. The conversion of **6** (TFA salt) in water for 48 h at room temperature followed by lyophilization yielded A $\beta$ 1–42 (**5**) quantitatively as TFA salt with >95% purity.

This result demonstrates that this '*O*-acyl isopeptide method' is applicable for the synthesis of large peptides. In particular, it is noteworthy that only one insertion of the isopeptide structure into the sequence of 42-residue

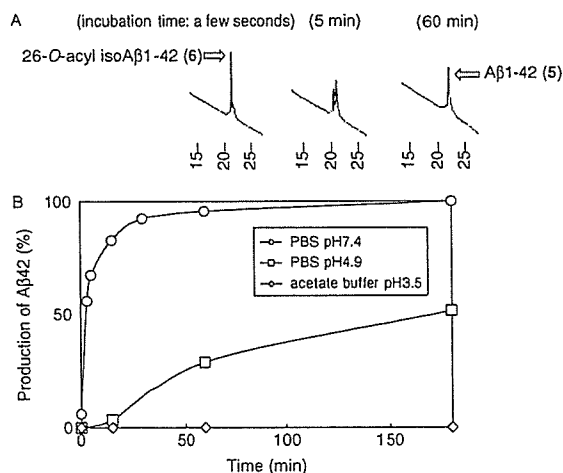
peptide can suppress the unfavourable nature of its difficult sequence. Therefore, the '*O*-acyl isopeptide method' can be applied to larger difficult sequence-containing peptides than A $\beta$ 1–42 as a general method. In addition, rapid migration of *O*-acyl isopeptides to intact A $\beta$ 1–42 under physiological conditions (pH 7.4) was observed while it was stable under storage conditions. Hence, our strategy not only overcomes the solubility problem in the synthesis of A $\beta$ 1–42, but also provides a novel tool for the biological evaluation system in AD research, in which 26-*O*-acyl isoA $\beta$ 1–42 can be stored in a solubilized form before use and rapidly produces intact monomer A $\beta$ 1–42 *in situ* during biological experiments.

### Acknowledgements

This research was supported in part by the 'Academic Frontier' Project for Private Universities: matching fund subsidy from MEXT (Ministry of Education, Culture, Sports, Science and Technology), and the 21st Century Center of Excellence Program 'Development of Drug Discovery Frontier Integrated from Tradition to Proteome' from MEXT. Y. S. is grateful for Research Fellowships of JSPS for Young Scientists. We are grateful to Mr T. Hamada for mass spectra measurements.

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**Figure 7** (A) HPLC profiles of the conversion of 26-AIA $\beta$ 42 (**6**) to A $\beta$ 1–42 (**5**) via *O*-*N* intramolecular acyl migration in PBS (pH 7.4) at 25 °C and (B) a graph of the production of A $\beta$ 1–42 (**5**) in various pH conditions at 25 °C. Analytical HPLC was performed using a C18 reverse-phase column (4.6 × 150 mm; YMC Pack ODS AM302) with a binary solvent system: the linear gradient of CH<sub>3</sub>CN (0–100% CH<sub>3</sub>CN for 40 min) in 0.1% aqueous TFA at a flow rate of 0.9 ml min<sup>-1</sup> (temperature: 40 °C), detected at 230 nm.



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## 'O-Acyl isopeptide method' for the efficient preparation of amyloid $\beta$ peptide 1–42 mutants

Youhei Sohma, Yousuke Chiyomori, Maiko Kimura, Fukue Fukao, Atsuhiko Taniguchi, Yoshio Hayashi,\* Tooru Kimura and Yoshiaki Kiso\*

Department of Medicinal Chemistry, Center for Frontier Research in Medicinal Science, 21st Century COE Program, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607-8412, Japan

Received 3 June 2005; revised 13 June 2005; accepted 14 June 2005  
Available online 19 August 2005

**Abstract**—Novel water-soluble isopeptides of A $\beta$ 1–42 mutants, '26-O-acyl isoA $\beta$ 1–42 (26-A1A $\beta$ 42) mutants', which were efficiently converted to intact A $\beta$ 1–42 mutants with no byproduct formation under physiological conditions, were synthesized. These isopeptides provide a new system useful for investigating the biological function of A $\beta$ 1–42 mutants.  
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### 1. Introduction

Amyloid  $\beta$  peptides (A $\beta$ s) are the main proteinaceous component of amyloid plaques found in the brain as a pathognomonic feature of Alzheimer's disease (AD),<sup>1</sup> and have been found to be neurotoxic in vivo and in vitro.<sup>2</sup> Although the predominant forms of A $\beta$  mainly consist of 40- and 42-residue peptides, designated A $\beta$ 1–40 and A $\beta$ 1–42, respectively, A $\beta$ 1–42 is thought to play a more critical role in amyloid formation and the pathogenesis of AD than A $\beta$ 1–40.<sup>3</sup> In addition, not only wild-type A $\beta$ 1–42 (D<sup>1</sup>AEFRHDSGY<sup>10</sup>EVHHQKLVF<sup>20</sup>F<sup>20</sup>AEDVGSNKG<sup>30</sup>IIGLMVGGVV<sup>40</sup>IA) observed in AD, but missense mutations inside the A $\beta$ -coding region in the amyloid precursor protein (APP) gene are also well-known. These mutations, known as Flemish-(A21G),<sup>4</sup> Arctic-(E22G),<sup>5</sup> Dutch-(E22Q),<sup>6</sup> Italian-(E22K),<sup>7</sup> and Iowa-type (D23N)<sup>8</sup> are found at positions 21–23 of A $\beta$ . All result in cerebral amyloid angiopathy (CAA) and/or cerebral parenchymal amyloidosis, leading to AD-like diseases. Recently, a Japanese-Tottori-type (D7N) mutation was also reported.<sup>9</sup> Recent studies have discussed several differences among A $\beta$  mutants in amyloid formation, metabolism, and elimination, which are related to the progression of AD-like diseases.<sup>10</sup>

Hence, more detailed studies comparing these features among A $\beta$  mutants would afford crucial information for understanding the mechanism of the diseases. A sufficient supply of synthetic A $\beta$  mutants would be key to this research.

Numerous studies have supported the hypothesis that neurotoxicity and the kinetics of A $\beta$ 1–42 aggregation are directly related to the assembly state in solution. However, the pathological self-assembly of A $\beta$ 1–42 in amyloid plaque formation, a currently unexplained process, is very difficult to demonstrate in vitro due to its uncontrolled polymerization. For example, synthesized A $\beta$ 1–42 already contains variable oligomeric forms,<sup>3d,11</sup> as A $\beta$ 1–42 undergoes time- and concentration-dependent aggregation in an aqueous TFA–acetonitrile solution used in HPLC purification.<sup>12</sup> Moreover, the A $\beta$ 1–42 monomer easily forms an aggregate even in a standard storage solution such as dimethylsulfoxide (DMSO).<sup>13</sup> Uncontrolled self-assembly in an in vitro experiment might cause considerable discrepancy in the biological data.<sup>3d,11</sup> Therefore, this highly agglutinative feature of A $\beta$ 1–42 is a significant obstacle for establishing a reliable in vitro biological experiment system to investigate the major causative agents of AD-like diseases.

The highly agglutinative property of A $\beta$ 1–42 in various media also results in synthetic difficulties with this peptide,<sup>12,14</sup> a so-called 'difficult sequence-containing peptide'.<sup>15</sup> In particular, in conventional reverse-phase HPLC purification of synthesized A $\beta$ 1–42 with the

**Keywords:** O-Acyl isopeptide method; Alzheimer's disease; A $\beta$ 1–42; A $\beta$ 1–42 mutant; 26-A1A $\beta$ 42; O-N intramolecular acyl migration reaction.

\* Corresponding authors. Tel.: +81 75 595 4635; fax: +81 75 591 9900; e-mail: [kiso@mb.kyoto-phu.ac.jp](mailto:kiso@mb.kyoto-phu.ac.jp)

aqueous TFA–acetonitrile system, it is too laborious to remove impurities accumulated during solid-phase peptide synthesis (SPPS) due to its low solubility and broad elution under either acidic or neutral conditions.

An ‘in situ’ system that prepares an intact monomer A $\beta$ 1–42 under physiological experimental conditions while suppressing the spontaneous self-assembly of A $\beta$ 1–42 under storage conditions would be advantageous in understanding the inherent pathological functions of agglutinative A $\beta$ 1–42 in AD-like diseases. For this purpose, based on the ‘O-acyl isopeptide method’,<sup>16</sup> we developed a novel water-soluble isopeptide of wild-type A $\beta$ 1–42, ‘26-O-acyl isoA $\beta$ 1–42’ (26-AIA $\beta$ 42, 8, Fig. 1).<sup>17</sup> This isopeptide exhibited higher water solubility than A $\beta$ 1–42 (1), and O–N intramolecular acyl migration reaction<sup>18,19</sup> to the original 1 occurred quickly with no side reaction under physiological conditions (pH 7.4), while 8 was stable under storage conditions.

We herein expand the ‘O-acyl isopeptide method’ to the synthesis of novel water-soluble O-acyl isopeptides

(9–14) of A $\beta$ 1–42 mutants, such as Japanese-Tottori (D7N, 2), Flemish-(A21G, 3), Arctic-(E22G, 4), Dutch-(E22Q, 5), Italian-(E22K, 6), and Iowa-type (D23N, 7). These synthesized isopeptides afforded each A $\beta$ 1–42 mutant under physiological conditions (Fig. 1) via O–N intramolecular acyl migration, providing a useful new system for investigating the biological function of A $\beta$ 1–42 mutants.

## 2. Chemistry

The synthetic scheme of Dutch-type 26-AIA $\beta$ 42 (E22Q, 12), based on the O-acyl isopeptide method, is depicted in Scheme 1 as a representative example. Fmoc-Ala-O-chlorotriethyl resin (15) was employed according to our previous study.<sup>17c</sup> Fmoc-protected amino acids were sequentially coupled using the DIPCDI (1,3-diisopropylcarbodiimide)–HOBT (1-hydroxybenzotriazole) method (2 h) after removing each Fmoc group with 20% piperidine–DMF (20 min) to give peptide resin 16. Then, Boc-Ser-OH was introduced to 16 by the DIPCDI–HOBT

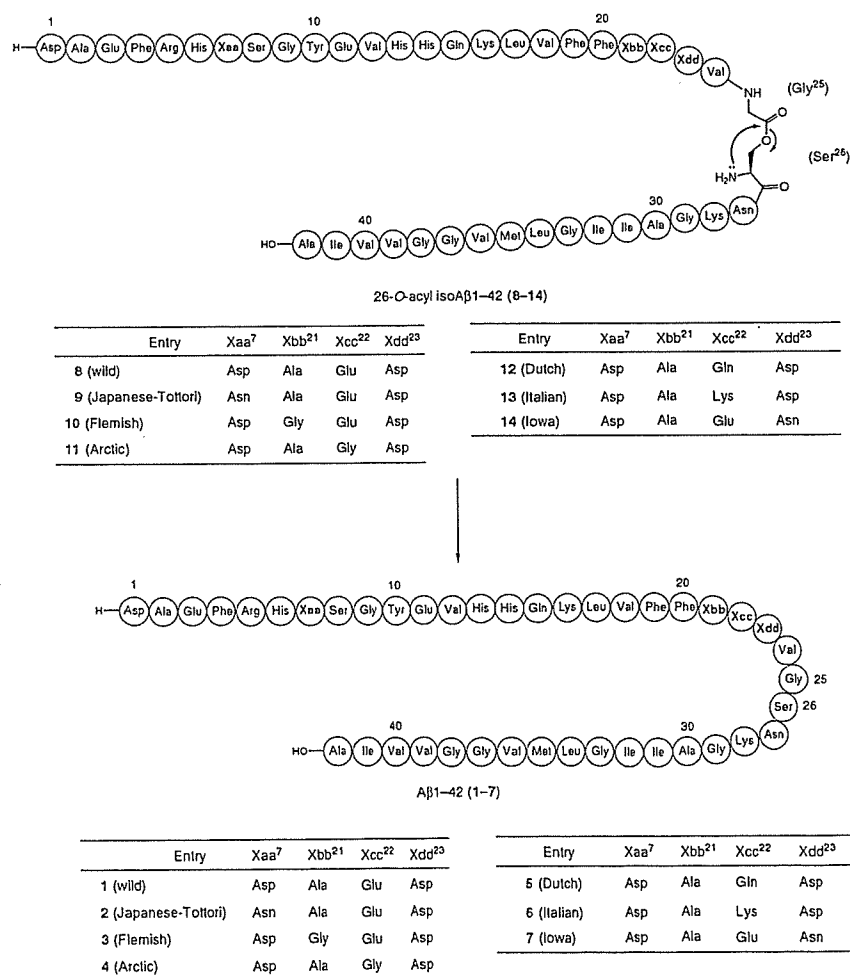
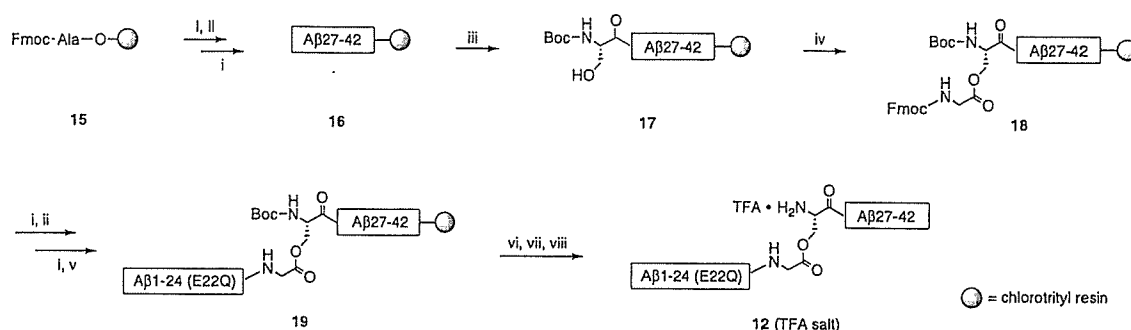


Figure 1. ‘O-Acyl isopeptide method’ for the efficient preparation of A $\beta$ 1–42 (1–7): the production of A $\beta$ 1–42 (1–7) via O–N intramolecular acyl migration of 26-O-acyl isoA $\beta$ 1–42 (8–14).



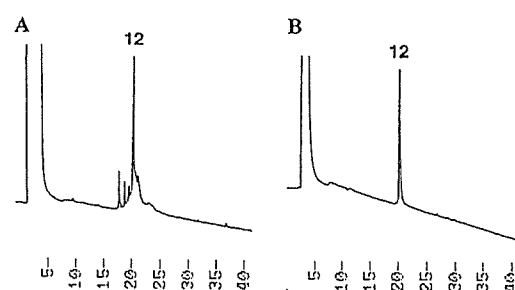
**Scheme 1.** Reagents and conditions: (i) 20% piperidine/DMF, 20 min; (ii) Fmoc-AA-OH (2.5 equiv), DIPCPI (2.5 equiv), HOBt (2.5 equiv), DMF, 2 h; (iii) Boc-Ser-OH (2.5 equiv), DIPCPI (2.5 equiv), HOBt (2.5 equiv), DMF, 2 h; (iv) Fmoc-Gly-OH (15.0 equiv), DIPCPI (15.0 equiv), DMAP (0.3 equiv),  $\text{CH}_2\text{Cl}_2$ , 4 h  $\times$  2; (v) Boc-Asp(OtBu)-OH (2.5 equiv), DIPCPI (2.5 equiv), HOBt (2.5 equiv), DMF, 2 h; (vi) TFA/*m*-cresol/thioanisole/ $\text{H}_2\text{O}$  (92.5:2.5:2.5:2.5), 90 min; (vii)  $\text{NH}_4\text{I}$  (20 equiv), dimethylsulfide (20 equiv), TFA/ $\text{H}_2\text{O}$  (2:1), 60 min, 0 °C; (viii) preparative HPLC (the linear gradient of  $\text{CH}_3\text{CN}$  in 0.1% aqueous TFA).

method (2 h) to obtain 17, which was coupled with Fmoc-Gly-OH at the  $\beta$ -hydroxy group of Ser using the DIPCPI-DMAP method<sup>18b</sup> in  $\text{CH}_2\text{Cl}_2$  to obtain ester 18. A protected Dutch-type 26-AIA $\beta$ 42 resin (19) was obtained through the subsequent coupling of amino acid residues using the conventional manner. Finally, Dutch-type 26-AIA $\beta$ 42 (12) was obtained as a major product (Fig. 2A) by treating 19 with TFA/*m*-cresol/thioanisole/ $\text{H}_2\text{O}$  (92.5:2.5:2.5:2.5) for 90 min followed by reduction with  $\text{NH}_4\text{I}$ -dimethylsulfide for 60 min in TFA/ $\text{H}_2\text{O}$  (2:1). Other 26-AIA $\beta$ 42 mutants (9–11, 13, and 14) were synthesized in a similar manner to that described in 12.

### 3. Results and discussion

In the HPLC analysis of crude Dutch-type isopeptide 12 (Fig. 2A), truncated peptide A $\beta$ 1–25 (DAEFRHDSG YEVHHQKLVFFAQDVG) was not detected as a by-product, although a small amount (1.8%, HPLC yield) of A $\beta$ 26–42 (SNKGAIIGLMVGGVVIA) was observed. This result indicates that the formed ester bond between Gly<sup>25</sup> and Ser<sup>26</sup> was almost stable in both 20% piperidine and TFA treatments, comparable to the synthesis of wild-type 26-AIA $\beta$ 42 (8), in which 1.6% of A $\beta$ 26–42 was detected in a crude sample.<sup>17</sup> The slight formation of A $\beta$ 26–42 observed in crude 12 might be attributed to dike-topiperazine formation during Fmoc group deprotection of Val<sup>24</sup> with 20% piperidine. Additionally, in the previous synthesis of 8 using the *O*-acyl isopeptide method, we confirmed that no side reaction occurred in the Boc-Ser-OH insertion (step (iii) in Scheme 1) or esterification (step (iv) in Scheme 1).<sup>17c</sup> The crude HPLC profiles of other 26-AIA $\beta$ 42 mutants (9–11, 13, and 14) were similar to that of 12.

The crude Dutch-type *O*-acyl isopeptide 12 was dissolved in DMSO, applied to preparative HPLC and eluted using 0.1% aqueous TFA- $\text{CH}_3\text{CN}$ . Since 12 was eluted as a sharp single peak (Figs. 2 and 3A), it was easily purified to give pure 12 (Figs. 2B and 3A) as a TFA salt with a total synthetic yield of 20.0%, calculated from the original loading onto the chlorotrityl resin. In addition, during the synthesis and purification



**Figure 2.** HPLC profiles of (A) crude and (B) purified Dutch-type 26-AIA $\beta$ 42 (12). Analytical HPLC was performed using a C18 reverse-phase column (4.6  $\times$  150 mm; YMC Pack ODS AM302) with a binary solvent system: the linear gradient of  $\text{CH}_3\text{CN}$  (0–100%  $\text{CH}_3\text{CN}$  for 40 min) in 0.1% aqueous TFA at a flow rate of 0.9 mL min<sup>-1</sup> (temperature: 40 °C), detected at 230 nm.



**Figure 3.** HPLC profiles of pure (A) Dutch-type 26-AIA $\beta$ 42 (12) and (B) Dutch-type A $\beta$ 1–42 (5). The same quantity (2.3 mmol) of both peptides was applied to the HPLC column as a DMSO solution. Analytical HPLC was performed using a C18 reverse-phase column (4.6 mm  $\times$  150 mm; YMC Pack ODS AM302) with a binary solvent system: the linear gradient of  $\text{CH}_3\text{CN}$  (25–55%  $\text{CH}_3\text{CN}$  for 60 min) in 0.1% aqueous TFA at a flow rate of 0.9 mL min<sup>-1</sup> (temperature: 40 °C), detected at 230 nm.

of 12, no conversion to Dutch-type A $\beta$ 1–42 (5) was observed. Similarly, the synthesis of other isopeptides 9–11, 13, and 14 was performed efficiently with a synthetic yield of 21.3–34.0% (see Section 5). These yields were higher than those obtained in the synthesis of A $\beta$ 1–42 mutants 2–7 by standard Fmoc-based SPPS (see Section 5). Since each 2–7 was eluted as a broad

Table 1. Water solubility of 26-A1A $\beta$ 42 (8–14) and A $\beta$ 1–42 (1–7)

26-A1A $\beta$ 42	A $\beta$ 1–42	Water solubility		
		26-A1A $\beta$ 42 (mg mL <sup>-1</sup> )	A $\beta$ 1–42 (mg mL <sup>-1</sup> )	ratio <sup>b</sup>
8 <sup>a</sup>	1 <sup>a</sup> (wild-type)	15.0	0.14	107
9	2 (Japanese-Tottori-type)	4.4	0.15	29.3
10	3 (Flemish-type)	18.3	0.95	19.3
11	4 (Arctic-type)	5.1	0.40	12.8
12	5 (Dutch-type)	14.5	0.93	15.6
13	6 (Italian-type)	16.2	8.7	1.9
14	7 (Iowa-type)	8.1	2.2	3.7

<sup>a</sup> Data from Ref. 17.

<sup>b</sup> Ratio = solubility of 26-A1A $\beta$ 42/solubility of A $\beta$ 1–42.

peak in preparative scale HPLC purification, it was laborious to separate the impurities and evaluate the purity as wild-type A $\beta$ 1–42 (1).<sup>14,17</sup>

The water solubility of mutant 26-A1A $\beta$ 42s (TFA salt) was 15.0 mg mL<sup>-1</sup> (8),<sup>17</sup> 4.4 mg mL<sup>-1</sup> (9), 18.3 mg mL<sup>-1</sup> (10), 5.1 mg mL<sup>-1</sup> (11), 14.5 mg mL<sup>-1</sup> (12), 16.2 mg mL<sup>-1</sup> (13), and 8.1 mg mL<sup>-1</sup> (14), higher than those in the corresponding mutant A $\beta$ 1–42s (1: 0.14,<sup>17</sup> 2: 0.15, 3: 0.95, 4: 0.40, 5: 0.93, 6: 8.7, and 7: 2.2 mg mL<sup>-1</sup>) (Table 1). The water solubility ratios of isopeptides compared to the corresponding A $\beta$ 1–42s were in a relatively wide range (1.9- to 107-fold), but the observed water solubility of each isopeptide was similar over the range of 4.4–18.3 mg mL<sup>-1</sup> (Table 1). This observation corresponds to our previous study regarding 'O-N intramolecular acyl/aclyoxy migration'-type water-soluble prodrugs of taxoids.<sup>19i</sup> It is suggested that the solubility of isopeptides is related much more to the isopeptide structure than to the amino acid substitution in each mutant, while the solubility of each A $\beta$ 1–42 is highly dependent on the nature of the mutated amino acid. In addition, the HPLC analysis of Dutch-type isopeptide 12 exhibited quite a sharp peak even in the slow gradient system (25–55% CH<sub>3</sub>CN, 60 min, Fig. 3A), while the corresponding A $\beta$ 1–42 5 was eluted as a broad peak under the same elution conditions (Fig. 3B) as wild-type A $\beta$ 1–42 1.<sup>14,17</sup> These results indicate that the highly insoluble and agglutinative nature of A $\beta$ 1–42 based on its secondary structure was suppressed by only one insertion of the isopeptide structure with a branched ester bond. A similar result was demonstrated in the case of wild-type A $\beta$ 1–42 1 in our previous study.<sup>17</sup> Therefore, the O-acyl isopeptide method is a common strategy for increasing water-solubility. Recent reports by Carpino et al.<sup>14i</sup> and Mutter et al.<sup>20</sup> have supported our data that O-acyl isopeptide structures have attractive solubilizing efficacy.

On the other hand, as shown in Figures 4 and 5B, Dutch-type 26-A1A $\beta$ 42 12 was quantitatively converted to the corresponding A $\beta$ 1–42 5 in phosphate-buffered saline (PBS, pH 7.4) at 37 °C with a half-life of ~1 min with no side reaction such as hydrolysis of the ester bond, and migration was completed after 30 min. An HPLC retention time (0–100% CH<sub>3</sub>CN for 40 min, 230 nm) of the newly appeared peak (Fig. 4) was identical to that of an independently synthesized Dutch-type A $\beta$ 1–42 5 by a conventional method. Moreover, the

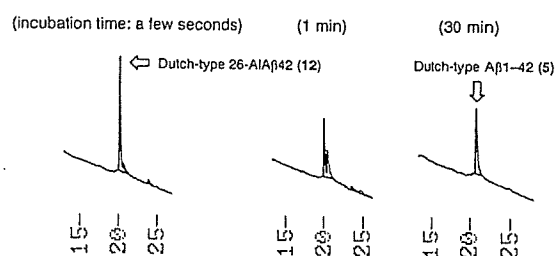


Figure 4. HPLC profiles of the conversion of Dutch-type 26-A1A $\beta$ 42 (12) to corresponding A $\beta$ 1–42 (5) in PBS (pH 7.4) at 37 °C. Analytical HPLC was performed using a C18 reverse-phase column (4.6 mm × 150 mm; YMC Pack ODS AM302) with a binary solvent system: the linear gradient of CH<sub>3</sub>CN (0–100% CH<sub>3</sub>CN for 40 min) in 0.1% aqueous TFA at a flow rate of 0.9 mL min<sup>-1</sup> (temperature: 40 °C), detected at 230 nm.

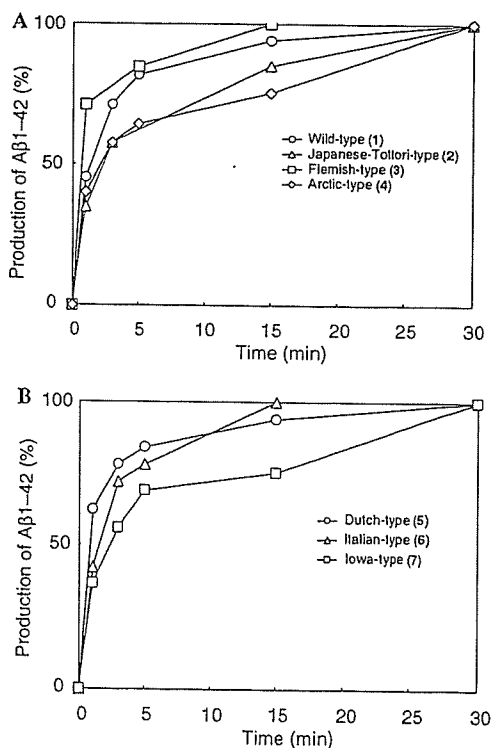


Figure 5. Time course of A $\beta$ 1–42 production in (A) wild-, Japanese-Tottori-, Flemish-, and Arctic-type isopeptides (B) Dutch-, Italian-, and Iowa-type isopeptides in PBS (pH 7.4) at 37 °C.

mass spectrometry analysis of this peak was identical to 5 ( $M_{\text{calcd}}$ : 4513.1;  $M+H_{\text{found}}$ : 4514.0). The rapid migration in 12 may be attributed to the lower steric hindrance of the Gly<sup>25</sup> residue. In contrast, the TFA salt of 12 was stable at 4 °C for at least 1 month in either the solid state or DMSO solution. Moreover, other mutant isopeptides (9–11, 13, and 14) were quantitatively converted to each A $\beta$ 1–42 mutant (for 2:  $M_{\text{calcd}}$ : 4513.1;  $M+H_{\text{found}}$ : 4514.1; for 3:  $M_{\text{calcd}}$ : 4500.0;  $M+H_{\text{found}}$ : 4501.4; for 4:  $M_{\text{calcd}}$ : 4442.0;  $M+H_{\text{found}}$ : 4443.0; for 6:  $M_{\text{calcd}}$ : 4513.1;  $M+H_{\text{found}}$ : 4513.9; for 7:  $M_{\text{calcd}}$ : 4513.1;  $M+H_{\text{found}}$ : 4514.2) at pH 7.4 (37 °C) in a short time with no significant difference in the half-life values (Fig. 5), indicating that amino acid substitutions at positions 21, 22, and 23, which are relatively far from position 26, do not significantly affect the migration rate at Gly<sup>25</sup>-Ser<sup>26</sup>. These rapid migrations under physiological conditions would enable the production of intact monomer A $\beta$ 1–42 mutants 'in situ' to investigate the inherent biological functions of A $\beta$ 1–42 mutants in AD-like diseases.

#### 4. Conclusion

The 'O-acyl isopeptide method' was successful in the efficient preparation of 26-O-acyl isopeptides of six A $\beta$ 1–42 mutants. Namely, the isopeptides (1) suppressed the unfavorable nature of A $\beta$ 1–42 mutants with only one insertion of the isopeptide structure into the whole sequence of the 42-residue peptide, and (2) could migrate to the corresponding A $\beta$ 1–42 mutants in a short time with no side reaction under physiological conditions (pH 7.4), while being stable under storage conditions. It is noteworthy that this method does not cause any negative effects by water-solubilizing auxiliaries in the biological experiment system, since no additional auxiliaries are released during conversion to A $\beta$ 1–42. These results suggest that this method provides a new system in AD-related research, in which 26-O-acyl isoA $\beta$ 1–42 (26-AIA $\beta$ 42) can be stored in a solubilized form and rapidly produce intact A $\beta$ 1–42 in situ during biological experiments, which is useful for investigating the biological function of A $\beta$ 1–42 mutants.

#### 5. Experimental

##### 5.1. General

All protected amino acids and resins were purchased from Calbiochem–Novabiochem Japan Ltd (Tokyo). Other chemicals were mainly purchased from commercial suppliers, Wako Pure Chemical Ind. Ltd (Osaka, Japan), Nacalai Tesque (Kyoto, Japan), and Aldrich Chemical Co. Inc. (Milwaukee, WI) and were used without further purification. MALDI-TOF MASS spectra were recorded on Voyager DE-RP using  $\alpha$ -cyano-4-hydroxycinnamic acid as a matrix. Analytical HPLC was performed using a C18 reverse-phase column (4.6 mm  $\times$  150 mm; YMC Pack ODS AM302) with a binary solvent system: a linear gradient of CH<sub>3</sub>CN in 0.1% aqueous TFA at a flow rate of 0.9 mL min<sup>-1</sup> (temperature: 40 °C), detected at 230 nm.

Preparative HPLC was carried out on a C18 reverse-phase column (20 mm  $\times$  250 mm; YMC Pack ODS SH343-5) with a binary solvent system: a linear gradient of CH<sub>3</sub>CN in 0.1% aqueous TFA at a flow rate of 5.0 mL min<sup>-1</sup> (temperature: 40 °C), detected at 230 nm. Solvents used for HPLC were of HPLC grade.

##### 5.2. Solid-phase peptide synthesis

The Fmoc-amino acid side-chain protections were selected as follows: *t*Bu (Asp, Glu, Ser, Thr, Tyr), Boc (Lys), Pmc (Arg), Trt (Asn, Gln, His). Generally, the peptide chains were assembled by the sequential coupling of activated *N*<sup>α</sup>-Fmoc-amino acid (2.5 equiv) in DMF (1.5–2 mL) in the presence of DIPCDI (2.5 equiv) and HOBt (2.5 equiv) with a reaction time of 2 h at room temperature. The resins were then washed with DMF (1.5 mL, 5 $\times$ ) and the completeness of each coupling was verified by the Keiser test. *N*<sup>α</sup>-Fmoc deprotection was carried out by treatment with piperidine (20% v/v in DMF) (2 mL, 1 min  $\times$  1 and 20 min  $\times$  1), followed by washing with DMF (1.5 mL, 10 $\times$ ) and chloroform (1.5 mL, 5 $\times$ ). If necessary, these coupling and deprotection cycles were repeated. After complete elongation of the peptide chains, the peptide resins were washed with methanol (1.5 mL, 5 $\times$ ) and dried for at least 2 h in vacuo. The peptides were then cleaved from the resin with TFA in the presence of thioanisole, *m*-cresol, and distilled water (92.5:2.5:2.5:2.5) for 90 min at room temperature, concentrated in vacuo, and precipitated with diethyl ether (4–8 mL) at 0 °C followed by centrifugation at 3000 rpm for 5 min (3 $\times$ ). The resultant peptides were dissolved or suspended in water and lyophilized for at least 12 h. The peptides were reduced using NH<sub>4</sub>I–dimethylsulfide in TFA/H<sub>2</sub>O (2:1) for 60 min at 0 °C, followed by concentration in vacuo. The crude products were purified by preparative reverse-phase HPLC with a 0.1% aqueous TFA–CH<sub>3</sub>CN system as an eluant, immediately frozen at –78 °C and lyophilized for at least 12 h. Purified peptides were stored dry at –20 °C until use.

##### 5.3. Amyloid $\beta$ peptide (A $\beta$ )1–42 (E22Q, 5) by 'O-acyl isopeptide method'

###### (1) 26-O-Acyl isoA $\beta$ 1–42(E22Q, 12)

After protected A $\beta$ 27–42-resin (chlorotriyl chloride resin, 0.1 mmol) was synthesized by the conventional method (see: conventional method for synthesizing 5), Boc-Ser-OH (49.2 mg, 0.24 mmol) was coupled by the DIPCDI (37.1  $\mu$ L, 0.24 mmol)–HOBt (32.1 mg, 0.24 mmol) method for 2 h in DMF (1.5 mL). Fmoc-Gly-OH (423.5 mg, 1.4 mmol) was coupled using the DIPCDI (223  $\mu$ L, 1.4 mmol)–DMAP (3.5 mg, 0.03 mmol) method in CH<sub>2</sub>Cl<sub>2</sub> (1.5 mL) for 4 h<sup>18b</sup> (2 $\times$ ). Subsequent amino acid residues were coupled after removing each Fmoc group using 20% piperidine for 20 min (resin: 521.2 mg). The resulting protected peptide resin was treated with TFA (10.3 mL)–*m*-cresol (261  $\mu$ L)–thioanisole (261  $\mu$ L)–H<sub>2</sub>O (261  $\mu$ L) for 90 min at rt, concentrated in vacuo, washed with diethyl ether, centrifuged, suspended in water, and lyophilized to give the crude O-acyl

isopeptide 12 (267.4 mg). This peptide 12 (10 mg) was dissolved in TFA (1 mL)–H<sub>2</sub>O (0.5 mL) in the presence of NH<sub>4</sub>I (6 mg, 0.04 mmol) and dimethylsulfide (3  $\mu$ L, 0.04 mmol) and stood for 60 min at 0 °C. After concentration in vacuo, the crude peptide was dissolved in DMSO, filtered using a 0.46  $\mu$ m filter unit, applied to preparative HPLC, and eluted using a 0.1% aqueous TFA–CH<sub>3</sub>CN. The desired fractions were collected and immediately lyophilized to afford peptide 12 as a white amorphous powder. Yield: 3.4 mg (20.0%); MALDI-MS (TOF):  $M_{\text{calcd}}$ : 4513.1;  $M+H_{\text{found}}$ : 4514.4; HPLC analysis at 230 nm: purity was >95%.

(2) The purified 12 was dissolved in PBS (pH 7.4) and stirred for 30 min at 37 °C to produce 5 quantitatively (determined by analytical HPLC). MALDI-MS (TOF):  $M_{\text{calcd}}$ : 4513.1;  $M+H_{\text{found}}$ : 4514.0; HPLC analysis at 230 nm: purity was >95%. The retention time on HPLC (0–100% CH<sub>3</sub>CN for 40 min, 230 nm) of 5 was identical to that of Dutch-type A $\beta$ 1–42 synthesized independently by the conventional method.

#### 5.4. A $\beta$ 1–42 (D7N, 2), (A21G, 3), (E22G, 4), (E22K, 6), (D23N, 7) by 'O-acyl isopeptide method'

(1) 26-O-Acyl isoA $\beta$ 1–42 (D7N, 9), (A21G, 10), (E22G, 11), (E22K, 13), (D23N, 14)

26-O-Acyl isoA $\beta$ 1–42 (9–11, 13, and 14) were synthesized in a similar manner to 12. Chemical data for 9: yield: 26.2%; MALDI-MS (TOF):  $M_{\text{calcd}}$ : 4513.1;  $M+H_{\text{found}}$ : 4514.5; HPLC analysis at 230 nm: purity was higher than 95%; for 10: yield: 26.4%; MALDI-MS (TOF):  $M_{\text{calcd}}$ : 4500.0;  $M+H_{\text{found}}$ : 4501.3; HPLC analysis at 230 nm: purity was higher than 95%; for 11: yield: 34.0%; MALDI-MS (TOF):  $M_{\text{calcd}}$ : 4442.0;  $M+H_{\text{found}}$ : 4442.9; HPLC analysis at 230 nm: purity was higher than 95%; for 13: yield: 24.6%; MALDI-MS (TOF):  $M_{\text{calcd}}$ : 4513.1;  $M+H_{\text{found}}$ : 4514.6; HPLC analysis at 230 nm: purity was higher than 95%; for 14: yield: 21.3%; MALDI-MS (TOF):  $M_{\text{calcd}}$ : 4513.1;  $M+H_{\text{found}}$ : 4514.5; HPLC analysis at 230 nm: purity was higher than 95%.

(2) The purified 9–11, 13, and 14 were dissolved in PBS (pH 7.4) and stirred for 30–60 min at 37 °C to produce 2–4, 6, and 7 quantitatively (determined by analytical HPLC). MALDI-MS (TOF): for 2:  $M_{\text{calcd}}$ : 4513.1;  $M+H_{\text{found}}$ : 4514.1; for 3:  $M_{\text{calcd}}$ : 4500.0;  $M+H_{\text{found}}$ : 4501.37; for 4:  $M_{\text{calcd}}$ : 4441.98;  $M+H_{\text{found}}$ : 4443.0; for 6:  $M_{\text{calcd}}$ : 4513.1;  $M+H_{\text{found}}$ : 4513.9; for 7:  $M_{\text{calcd}}$ : 4513.1;  $M+H_{\text{found}}$ : 4514.2; HPLC analysis of 2–4, 6, and 7 at 230 nm: purity was >95%. The retention time on HPLC (0–100% CH<sub>3</sub>CN for 40 min, 230 nm) of formed 2–4, 6, and 7 was identical to that of each independently synthesized A $\beta$ 1–42 mutant by the conventional manner.

#### 5.5. Amyloid $\beta$ peptide (A $\beta$ )1–42 (E22Q, 5) by the conventional method

The chlorotrityl chloride resin (200 mg, 0.3 mmol) and Fmoc-Ala-OH (49.4 mg, 0.15 mmol) were taken to the manual solid-phase reactor under an argon atmosphere and stirred for 2.5 h in the presence of *N,N*-diisopropyl-

ethylamine (DIPEA, 26.2  $\mu$ L, 0.15 mmol) in 1,2-dichloroethane (1.5 mL). After washing with DMF (1.5 mL, 5 $\times$ ), capping was performed with MeOH (200  $\mu$ L) in the presence of DIPEA (52.5  $\mu$ L, 0.3 mmol) in DMF for 20 min. After washing with DMF (1.5 mL, 5 $\times$ ), DMF–H<sub>2</sub>O (1:1, 1.5 mL, 5 $\times$ ), CHCl<sub>3</sub> (1.5 mL, 2 $\times$ ), and MeOH (1.5 mL, 2 $\times$ ) followed by drying in vacuo, the loading ratio was determined (0.04 mmol) photometrically from the amount of Fmoc chromophore liberated upon treatment with 50% piperidine–DMF for 30 min at 37 °C. The sequential Fmoc-protected amino acids (0.09 mmol) were manually coupled in the presence of DIPCDI (14.1  $\mu$ L, 0.09 mmol) and HOBT (12.2 mg, 0.09 mmol) for 2 h in DMF (1.5 mL) after the removal of each Fmoc group by 20% piperidine–DMF for 20 min (resin: 362 mg). The resulting protected peptide resin was treated with TFA (7.1 mL)–*m*-cresol (181  $\mu$ L)–thioanisole (181  $\mu$ L)–H<sub>2</sub>O (181  $\mu$ L) for 90 min at rt, concentrated in vacuo, washed with diethyl ether, centrifuged, suspended in water, and lyophilized to give the crude peptide 5 (141.2 mg). This peptide (10 mg) was dissolved in TFA (1 mL)–H<sub>2</sub>O (0.5 mL) in the presence of NH<sub>4</sub>I (6 mg, 0.04 mmol), and dimethylsulfide (3  $\mu$ L, 0.04 mmol), and stood for 60 min at 0 °C. After concentration in vacuo, the crude peptide was dissolved in DMSO, filtered using a 0.46  $\mu$ m filter unit, applied to preparative HPLC, and eluted using 0.1% aqueous TFA–CH<sub>3</sub>CN. The peak fractions were collected and immediately lyophilized to afford the desired peptide 5 as a white amorphous powder. Yield: 0.8 mg (7.2%); MALDI-MS (TOF):  $M_{\text{calcd}}$ : 4513.0;  $M+H_{\text{found}}$ : 4514.2; HPLC analysis at 230 nm: purity was >94%.

#### 5.6. A $\beta$ 1–42 (D7N, 2), (A21G, 3), (E22G, 4), (E22K, 6), (D23N, 7) by the conventional method

Peptides 2–4, 6, and 7 were synthesized in a similar manner to peptide 5. Chemical data for 2: yield: 8.1%; MALDI-MS (TOF):  $M_{\text{calcd}}$ : 4513.1;  $M+H_{\text{found}}$ : 4514.0; HPLC analysis at 230 nm: purity was higher than 94%; for 3: yield: 0.5%; MALDI-MS (TOF):  $M_{\text{calcd}}$ : 4500.0;  $M+H_{\text{found}}$ : 4501.4; HPLC analysis at 230 nm: purity was higher than 95%; for 4: yield: 10.1%; MALDI-MS (TOF):  $M_{\text{calcd}}$ : 4442.0;  $M+H_{\text{found}}$ : 4442.9; HPLC analysis at 230 nm: purity was higher than 95%; for 6: yield: 22.7%; MALDI-MS (TOF):  $M_{\text{calcd}}$ : 4513.1;  $M+H_{\text{found}}$ : 4514.2; HPLC analysis at 230 nm: purity was higher than 95%; for 7: yield: 6.7%; MALDI-MS (TOF):  $M_{\text{calcd}}$ : 4513.1;  $M+H_{\text{found}}$ : 4513.9; HPLC analysis at 230 nm: purity was higher than 95%. The elution profiles of A $\beta$ 1–42 mutants in a slow gradient system (25–55% CH<sub>3</sub>CN for 60 min) were ill-defined; however, TOF-MS indicated that only a single species was present. Glabe and coworkers<sup>14a</sup> and Johnson and coworkers<sup>14b</sup> have commented upon the chromatographic behavior of A $\beta$ , suggesting that the ill-defined profiles are intrinsic properties of the peptides.

#### 5.7. Water solubility

Peptides were saturated in distilled water and shaken at room temperature. The saturated solutions were passed through a centrifugal filter (0.46  $\mu$ m filter unit,



Ultrafree®-MC, Millipore). The filtrate was analyzed by RP-HPLC to determine its solubility.

#### 5.8. Stability of *O*-acyl isopeptides 9–14 in phosphate-buffered saline (pH 7.4)

To 495  $\mu$ L of PBS (pH 7.4) were added 5  $\mu$ L of a solution including each *O*-acyl isopeptide (1 mM in DMSO), and the mixture was incubated at 37 °C in a water bath. At the desired time points, 500  $\mu$ L of 1,1,1,3,3,3-hexafluoro-2-propanol was added to the samples and 500  $\mu$ L of the mixture was directly analyzed by RP-HPLC. HPLC was performed using a C18 (4.6  $\times$  150 mm; YMC Pack ODS AM302) reverse-phase column with a binary solvent system: linear gradient of CH<sub>3</sub>CN (0–100%, 40 min) in 0.1% aqueous TFA at a flow rate of 0.9 mL min<sup>-1</sup>, detected at UV 230 nm.

#### Acknowledgments

This research was supported in part by the 'Academic Frontier' Project for Private Universities: matching fund subsidy from MEXT (Ministry of Education, Culture, Sports, Science and Technology), and the 21st Century Center of Excellence Program 'Development of Drug Discovery Frontier Integrated from Tradition to Proteome' from MEXT. Y.S. is grateful for Research Fellowships of JSPS for Young Scientists. We thank Mr. T. Yoshiya, Ms. S. Nakamura, and Ms. M. Tanimiya for technical assistance. We are grateful to Mr. T. Hamada for mass spectra measurements.

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## Overcoming Roadblocks in Lead Optimization: A Thermodynamic Perspective

Adam J Ruben<sup>1</sup>, Yoshiaki Kiso<sup>2</sup> and Ernesto Freire<sup>1,\*</sup>

<sup>1</sup>Department of Biology, Johns Hopkins University, Baltimore, MD 21218, USA

<sup>2</sup>Department of Medicinal Chemistry, Center for Frontier Research in Medicinal Science, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607-8412, Japan

\*Corresponding author: Ernesto Freire, ef@jhu.edu

Received 6 October 2005, revised and accepted for publication 11 October 2005

One of the most important goals in the development of lead compounds is the optimization of their binding affinity towards the intended target, as binding affinity is directly related to potency. Moreover, this goal needs to be accomplished in ways that do not compromise important properties like solubility or bioavailability. Recent experimental and computational developments permit monitoring of the thermodynamic forces that define the binding affinity, the enthalpy and entropy changes, thus providing a rigorous guideline to the optimization process. Having access to individual components of the binding affinity rather than its overall value accelerates the optimization process and facilitates the achievement of extremely high affinity.

Binding affinity originates from different types of interactions between the drug molecule and the target protein as well as their interactions with the solvent (water). In the optimization of binding affinity, there are terms that can be controlled by the designer and terms that are beyond control. The loss of translational degrees of freedom, for example, cannot be altered. For competitive inhibitors that target the same site, the energy associated with conformational changes in the protein is usually constant and cannot be manipulated. The situation is obviously different for drugs that operate allosterically by modulating conformational changes. From the viewpoint of affinity optimization, the binding energy can be viewed as the difference between the interaction energy with the target and the desolvation energy of the drug molecule. These interactions contribute in a characteristic fashion to the enthalpy and entropy of binding, two quantities that can be measured experimentally by isothermal titration calorimetry (ITC) and that can be used to guide the optimization process.

At the thermodynamic level, the binding affinity is determined by the magnitude of the Gibbs energy ( $\Delta G$ ), which is a function of only two terms, the enthalpy ( $\Delta H$ ) and the entropy ( $\Delta S$ ) changes. As the enthalpy and entropy contribute to the binding energy in an additive

fashion ( $\Delta G = \Delta H - T\Delta S$ ), it is clear that an infinite number of enthalpy and entropy values can add up to yield the same Gibbs energy value. Compounds that exhibit the same  $\Delta G$  will bind to the target with the same affinity; however, compounds that are either predominantly enthalpic or entropic will differ in other aspects, as the enthalpy and entropy changes originate from different types of interactions.

The enthalpy change reflects the strength of drug/target interactions in relation to those with the solvent. The favorable term arises primarily from van der Waals and hydrogen bonding interactions between drug and target. Two major terms define the binding entropy; the first one is the solvation entropy associated with the burial from the solvent of hydrophobic groups, and the second one is the conformational entropy, which usually reflects the loss of conformational degrees of freedom upon binding. From the engineering point of view, a favorable enthalpy change is obtained from good geometric complementarity between drug and target and the proper location of hydrogen bond donors and acceptors. As these interactions are stereo-specific, a favorable enthalpy change is not only an important contributor to affinity but also to selectivity. The solvation entropy change reflects a repulsion of the drug from the solvent rather than an attractive interaction with the target. This is a favorable but non-specific force proportional to the hydrophobicity of the drug. The conformational entropy change, on the other hand, usually reflects a loss of conformational degrees of freedom in the drug molecule and protein, being therefore an unfavorable term. The magnitude of the conformational entropy loss can be reduced by introducing conformational constraints in the drug molecule so that it occupies similar conformations in the free and bound states.

### Affinity Optimization

The binding affinity of a compound can be improved by generating a favorable binding enthalpy, favorable solvation entropy, or by minimizing the unfavorable conformational entropy. Obviously, extremely high affinity is achieved when the three factors are optimized simultaneously. The degree of difficulty associated with optimizing the enthalpy is not the same as the one associated with optimizing the entropy. Historically, it has proven much easier to optimize the entropy. As the major favorable contributor is the hydrophobic effect, which is proportional to the number of non-polar groups that are buried from the solvent, the tendency throughout the years has been toward an increase in the hydrophobicity of drug candidates (1, 2). Medicinal chemists have long learned to conformationally constrain and pre-shape molecules to the geometry of the binding site, which completes the entropy optimization. According to these

**Table 1:** Desolvation enthalpy of different chemical functionalities at 25 °C.

Group	$\Delta H$ (kcal/mol)	Group	$\Delta H$ (kcal/mol)	Group	$\Delta H$ (kcal/mol)
NH <sub>2</sub>	7.9	OH	8.7	CH <sub>3</sub>	0.57
NH	9.4	SO	12.7	CH <sub>2</sub>	0.77
N	9.3	CO	5.5	CH	0.73
NO <sub>2</sub>	4.7	COO	5.4	CH <sub>aromatic</sub>	0.7
O	5.2	COOH	8.4	C	1.1

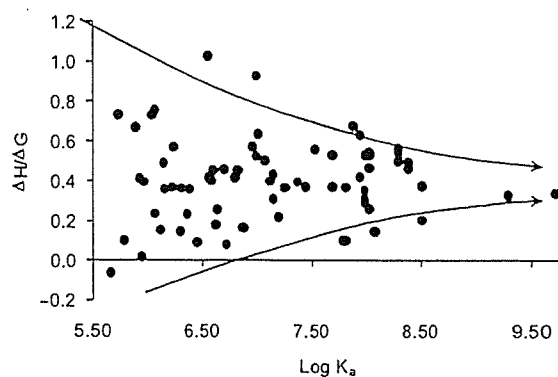
Values have been taken from Cabani *et al.* [9] and represent values from water to the gas phase.

traditional precepts, affinity is achieved through hydrophobicity, and selectivity is achieved through shape complementarity.

There is a limit, however, to the hydrophobic character that can be imparted to a compound before it becomes completely insoluble and useless as a drug molecule. At some point in the optimization process, it becomes necessary to introduce favorable enthalpic interactions if the goal is to achieve nanomolar or sub-nanomolar affinities. The exact threshold depends on the characteristics of the target site. Compounds that exhibit extremely high affinity have been shown to display both favorable entropic and enthalpic interactions (3–5). Despite the limits to affinity, a compound that derives selectivity primarily from shape complementarity is prone to lose some when confronted with homologous enzymes with structurally similar binding pockets.

Even though enthalpic interactions are required for extremely high affinity and improved selectivity, the optimization of the binding enthalpy has been notoriously more difficult than the optimization of the binding entropy, the reason being that the enthalpy of desolvation of polar groups is very large and unfavorable, as shown in Table 1. Polar groups carry a desolvation penalty about one order of magnitude larger than non-polar groups. A polar group needs to establish a very good interaction with the target in order to compensate for the desolvation penalty and make a favorable contribution. For this reason, they are often engineered as solubilizers of otherwise extremely hydrophobic compounds rather than major contributors to affinity.

As the major contributors to the binding enthalpy are polar groups, a common misconception is that enthalpically driven compounds must be highly polar and that consequently their bioavailability will be compromised. In fact, what is often observed experimentally is that compounds with the same number of polar groups have vastly different binding enthalpies. For example, among the HIV-1 protease inhibitors, saquinavir and TMC-126 have exactly the same number of polar groups; however, saquinavir binds to the protease with an unfavorable enthalpy of 1.5 kcal/mol, whereas TMC-126 does so with a very favorable binding enthalpy of  $-12$  kcal/mol. To generate a favorable binding enthalpy, it is not the number of polar groups that matters but the quality of their interactions with the target. It is better to have few groups that establish strong interactions than a large number of groups mostly paying the desolvation penalty. In fact, it has been shown that there is no correlation between the enthalpic character of a compound and the Lipinski rules of five (4).



**Figure 1:** Enthalpic contribution to the Gibbs energy of binding ( $\Delta H/\Delta G$ ) versus the logarithm of the binding affinity ( $\text{Log } K_d$ ) for 71 allophenylnorstatine inhibitors of plasmepsin II. All thermodynamic parameters were determined under identical conditions (10 mM formate buffer, pH 4.0, 2% DMSO, at 25 °C).

Fortunately, the situation is changing on two fronts. Experimentally, ITC permits monitoring of the enthalpy and entropy changes throughout the optimization process and therefore a direct evaluation of the thermodynamic consequences of introducing different functionalities at specific sites. At the computational level, the success of the initial work dealing with the derivation of empirical correlations between binding enthalpy and structural parameters (6) has led to new ways of predicting enthalpy from structure and of predicting the enthalpic effects expected from the introduction of different functionalities into a given scaffold.

## The optimization funnel

One of the ongoing projects in this laboratory is the development of plasmepsin inhibitors as new anti-malarial drugs (7). Starting with the allophenylnorstatine scaffold that mimics the main cleavage site in the hemoglobin molecule of infected victims, we have been able to generate high affinity inhibitors with  $K_i$ 's in the high picomolar range (7, 8). The evolution of the potency of these compounds reflects the situation encountered in most drug discovery laboratories when a given chemical scaffold begins to be optimized. Starting with hits characterized by  $K_i$ 's in the micromolar range, the goal is to increase potency by three to five orders of magnitude, i.e. an increase in the Gibbs energy of binding of 4–7 kcal/mol. How can this be achieved? How do the individual components of the Gibbs energy advance?

Figure 1 shows the evolution of the contribution of the enthalpy change to the total Gibbs energy of binding as the affinity of the compounds to plasmepsin II is optimized from the micromolar to the high picomolar level. It is immediately apparent that low affinity compounds can exhibit a wide range of enthalpy/entropy combinations. In other words, low affinity can be generated by essentially any combination of hydrophobic and polar interactions. As the affinity increases, the range of enthalpy/entropy combinations narrows