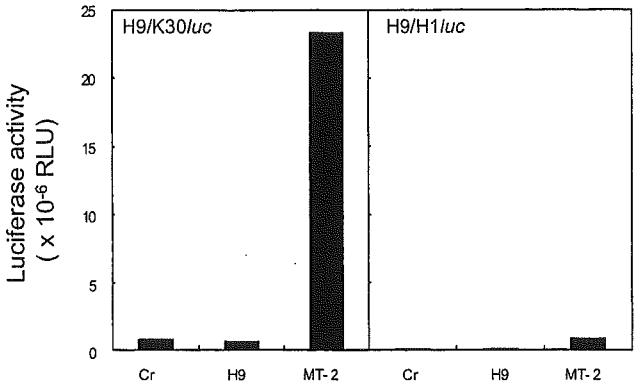

Fig. 2. Enhancement of luciferase production in co-cultures of indicator and MT-2 cells. Indicator cell lines H9/K30*luc* for HTLV-1 (this paper) and H9/H1*luc* for HIV-1 [14] (10^6) were co-cultured with H9 or HTLV-1 producer MT-2 cells (10^6), and 2 days later, cell lysates were prepared for luciferase assay. Cultures of indicator cells only served as Cr.

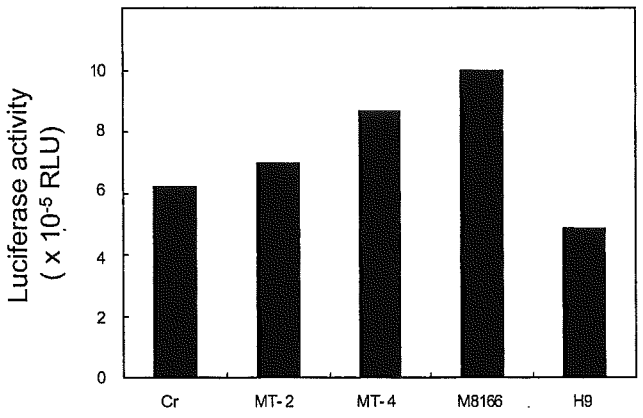

Fig. 3. Potential of cell-free virus from MT-2 to augment luciferase production in H9/K30*luc* cells. Cell-free culture supernatants were prepared from various cultures (MT-2, MT-4, M8166, and H9) maintained at growing phase for 2 days, and inoculated into the indicator cell line H9/K30*luc*. On the next day, cell lysates were prepared for luciferase assay. Culture of indicator cells only served as Cr. Cell lines MT-2 [19] and H9 [18] are HTLV-1-positive and -negative, respectively. Cell lines MT-4 [19] and M8166 [20] are HTLV-1 DNA-positive but negative for HTLV-1.

significant difference was observed among co-cultures of H9/K30*luc* and MT-2 cells.

3.2. Effects of saquinavir (SQV) on HTLV-1 and HIV-1 as determined by our luciferase system

Based on the results described above, we assumed that Tax transported from MT-2 to H9/K30*luc* cells by Env-mediated membrane fusion enhances the luciferase production, and that, if this process is suppressed, luciferase production is significantly reduced. It has been reported for HIV-1 recently that interactions between unprocessed Gag and the cytoplasmic tail of Env-gp41 suppress cell fusion [27]. We, therefore, checked by our system the effects of a protease inhibitor SQV on HIV-1 and HTLV-1. SQV has been reported to be very effective against HIV-1 protease but fails to inhibit HTLV-I Gag processing in infected cells [28]. To obtain appropriate HIV-1 producer cells, which are stably infected with HIV-

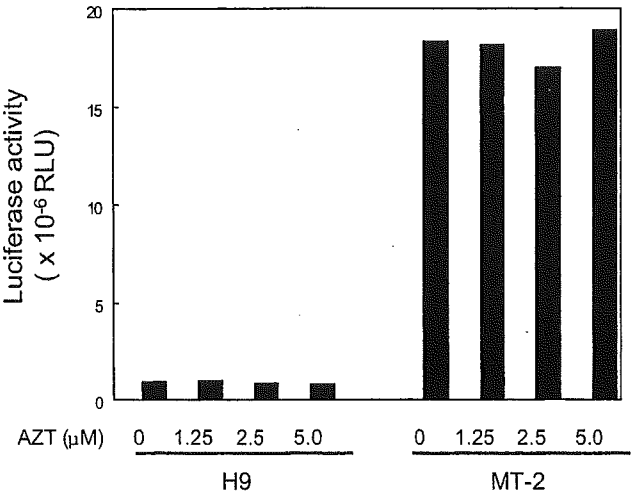

Fig. 4. Effect of AZT on luciferase production in the co-cultures of H9/K30*luc* and MT-2 cells. Indicator H9/K30*luc* cells (10^6) were co-cultured with H9 or HTLV-1 producer MT-2 cells (10^6) in the absence or presence of AZT as indicated for 48 h, and cell lysates were prepared for luciferase assay. H9 and MT-2 cells had been pre-cultured for 24 h in the absence or presence of AZT as above before co-culture started. No cytotoxic effects were observed in these conditions.

1 and producing a low level of the virus-like MT-2 cells for HTLV-1, H9 cells were electroporated with pNL432 and cultured for months. The resultant H9/NL432 cells were easily maintained, and produced a low level of HIV-1 as monitored by RT assay (data not shown).

By the use of H9/H1*luc*, H9/K30*luc*, H9/NL432, and MT-2 cells as indicator and virus producer cells, we determined the inhibitory effects of SQV on HIV-1 and HTLV-1 by monitoring luciferase activity. The effects of SQV on viability of cells were also determined to confirm that there would be no experimental error caused by cytotoxicity. As shown in Fig. 5, while luciferase production in the HIV-1 co-culture was severely inhibited by SQV, no appreciable effects were observed for the HTLV-1 co-culture. These data were in good agreement with our assumption and the results previously reported [27,28] as mentioned above.

4. Discussion

In this report, we have established an indicator cell line for HTLV-1 infection based on luciferase assay (Figs. 1 and 2). Although the cell line H9/K30*luc* was highly susceptible to infection by the co-culture method (Fig. 1), it was insensitive to infection with cell-free HTLV-1 (Fig. 3) as expected [15,16]. Enhanced production of luciferase observed in the co-cultures of H9/K30*luc* and MT-2 cells was probably due to the Tax already present in MT-2 cells before co-cultivation (Fig. 4). Therefore, we have concluded that our HTLV-1 system described here monitors the efficiency of Env-mediated membrane fusion, and that it is useful for evaluating the ability of various factors or agents affecting the process. Indeed, SQV was demonstrated to be a powerful inhibitor for HIV-1 by affecting cell fusion indirectly (Fig. 5).

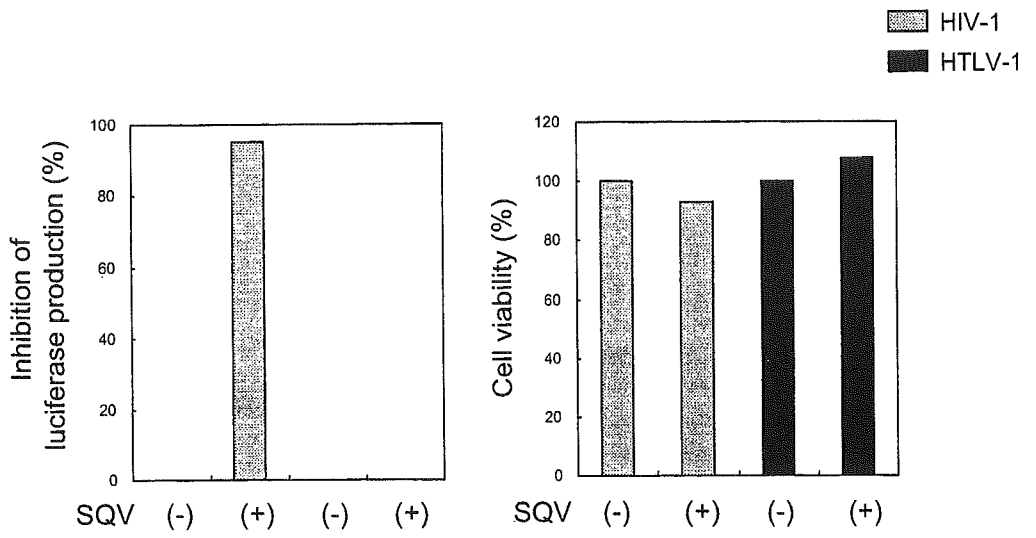

Fig. 5. Effects of SQV on the co-cultures of indicator and virus-producing cells. Indicator (H9/H1*luc* for HIV-1 and H9/K30*luc* for HTLV-1) and virus-producing cells (H9/NL432 for HIV-1 and MT-2 for HTLV-1) (10^6 for each) were co-cultured for 48 h in the absence (–) or presence of SQV (+, 2 μM), and cell lysates were prepared for luciferase assay. Data presented are relative to those of cultures without SQV. H9/NL432 and MT-2 cells had been pre-cultured for 24 h in the absence or presence of SQV as above before co-culture started. Cell viability was determined by the Cell Counting Kit-8, and relative values are presented.

Our results described here strongly suggest that our biological assay system can be used for screening of inhibitors against HTLV-1 and HIV-1 proteases. In particular, because HIV-1 protease inhibitors cannot be effective against HTLV-1 protease (Fig. 5) [28], and because no other good assay methods are available for HTLV-1, the screening by the H9/K30*luc*-MT-2 system would be important. Furthermore, pathogenesis of HAM/TSP can be controlled by reducing the level of HTLV-1 in infected individuals [5–13].

Our protocol for monitoring the inhibitory effects of potential protease inhibitors on HTLV-1 and HIV-1 is summarized as shown in Fig. 6. By using appropriate producer and indicator cells, a large number of antiviral agents can be checked readily for their ability to inhibit the replication of HTLV-1 and HIV-1 within days. Screening of various candidate protease inhibitors by the protocol in Fig. 6 is now in progress in our laboratory.

Virus-producer cells
↓
Culture in the presence of protease inhibitors for 24hrs
↓
Co-cultivation with indicator cells in the presence of protease inhibitors for 48hrs
↓
Assays for luciferase and cell viability

Virus	Producer cells	Indicator cells
HIV-1	H9/NL432	H9/H1*luc*
HTLV-1	MT-2	H9/K30*luc*

Fig. 6. Evaluation system for the effects of human retroviral protease inhibitors. Based on the results in this report, validity of human retroviral protease inhibitors can be readily evaluated as shown in this figure.

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References

[1] M. Yoshida, I. Miyoshi, Y. Hinuma, Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in the disease, Proc. Natl. Acad. Sci. USA 79 (1982) 2031–2035.

[2] A. Gessain, F. Barin, J.C. Vernant, O. Gout, L. Maurs, A. Calender, G. De The, Antibodies to human T-lymphotropic virus type I in patients with tropical spastic paraparesis, Lancet ii (1985) 407–410.

[3] M. Osame, K. Usuku, S. Izumo, N. Ijichi, H. Amitani, A. Igata, M. Matsumoto, M. Tara, HTLV-I associated myelopathy, a new clinical entity, Lancet i (1986) 1031–1032.

[4] K. Barmak, E. Harhaj, C. Grant, T. Alefantis, B. Wigdahl, Human T-cell leukemia virus type I-induced disease: pathways to cancer and neurodegeneration, Virology 308 (2003) 1–12.

[5] M. Osame, M. Matsumoto, K. Usuku, S. Izumo, N. Ijichi, H. Amitani, M. Tara, A. Igata, Chronic progressive myelopathy associated with

elevated antibodies to human T-lymphotropic virus type I and adult T-cell leukemialike cells, Ann. Neurol. 21 (1987) 117–122.
[6] M. Mori, K. Kinoshita, N. Ban, Y. Yamada, H. Shiku, Activated T-lymphocytes with polyclonal gammopathy in patients with human T-lymphotropic virus type I-associated myelopathy, Ann. Neurol. 24 (1988) 280–282.
[7] M. Yoshida, M. Osame, H. Kawai, M. Toita, N. Kuwasaki, Y. Nishida, Y. Hiraki, K. Takahashi, K. Nomura, S. Sonoda, N. Eiraku, S. Ijichi, K. Usuku, Increased replication of HTLV-I in HTLV-I-associated myelopathy, Ann. Neurol. 26 (1989) 331–335.
[8] S.J. Greenberg, S. Jacobson, T.A. Waldmann, D.E. McFarlin, Molecular analysis of HTLV-I proviral integration and T-cell receptor arrangement indicates that T cells in tropical spastic paraparesis are polyclonal, J. Infect. Dis. 159 (1989) 741–744.
[9] M. Nishimura, A. Adachi, I. Akiguchi, N. Shirahata, M. Maeda, A. Ishimoto, T. Mezaki, J. Kimura, High ratio of HTLV-1-infected cells in HTLV-1 associated myelopathy (HAM), Acta Neurol. Scand. 81 (1990) 209–214.
[10] K. Nagasato, T. Nakamura, K. Ohishi, K. Shibayama, M. Motomura, K. Ichinose, et al., Active production of anti-human T-lymphotropic virus type I (HTLV-I) IgM antibody in HTLV-I-associated myelopathy, J. Neuroimmunol. 32 (1991) 105–109.
[11] J. Kira, Y. Koyanagi, T. Yamada, Y. Itoyama, I. Goto, N. Yamamoto, H. Sasaki, Y. Sakaki, Increased HTLV-I proviral DNA in HTLV-I-associated myelopathy: a quantitative polymerase chain reaction study, Ann. Neurol. 29 (1991) 194–201.
[12] R. Kubota, T. Fujiyoshi, S. Izumo, S. Yashiki, I. Maruyama, M. Osame, H. Sasaki, Y. Sakaki, Fluctuation of HTLV-I proviral DNA in peripheral blood mononuclear cells of HTLV-I-associated myelopathy, J. Neuroimmunol. 42 (1993) 147–154.
[13] R. Kubota, F. Umehara, S. Izumo, S. Ijichi, K. Matsumoto, S. Yashiki, T. Fujiyoshi, S. Sonoda, M. Osame, HTLV-I proviral DNA amounts correlates with infiltrating CD4+ lymphocytes in the spinal cord from patients with HTLV-I-associated myelopathy, J. Neuroimmunol. 53 (1994) 23–29.
[14] T. Nagao, A. Yoshida, A. Sakurai, A. Piroozmand, A. Jere, M. Fujita, T. Uchiyama, A. Adachi, Determination of HIV-1 infectivity by lymphocytic cell lines with integrated luciferase gene, Int. J. Mol. Med. 14 (2004) 1073–1076.
[15] N. Fan, J. Gavalchin, B. Paul, K.H. Wells, M.J. Lane, B.J. Poiesz, Infection of peripheral blood mononuclear cells and cell lines by cell-free human T-cell lymphoma/leukemia virus type I, J. Clin. Microbiol. 30 (1992) 905–910.
[16] D. Derse, S.A. Hill, P.A. Lloyd, H.K. Chung, B.A. Morse, Examining human T-lymphotropic virus type 1 infection and replication by cell-free infection with recombinant virus vectors, J. Virol. 75 (2001) 8461–8468.
[17] J.S. Lebkowski, S. Clancy, M.P. Calos, Simian virus 40 replication in adenovirus-transformed human cells antagonizes gene expression, Nature 317 (1985) 169–171.
[18] D.L. Mann, S.J. O'Brien, D.A. Gilbert, Y. Reid, M. Popovic, E. Read-Connole, R.C. Gallo, A.F. Gazdar, Origin of the HIV-susceptible human CD4+ cell line H9, AIDS Res. Hum. Retroviruses 5 (1989) 253–255.
[19] S. Harada, Y. Koyanagi, N. Yamamoto, Infection of HTLV-III/LAV in HTLV-I-carrying cells MT-2 and MT-4 and application in a plaque assay, Science 229 (1985) 563–566.
[20] R. Shibata, M. Kawamura, H. Sakai, M. Hayami, A. Ishimoto, A. Adachi, Generation of a chimeric human and simian immunodeficiency virus infectious to monkey peripheral blood mononuclear cells, J. Virol. 65 (1991) 3514–3520.
[21] A. Adachi, H.E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, M.A. Martin, Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone, J. Virol. 59 (1986) 284–291.
[22] R.L. Willey, D.H. Smith, L.A. Lasky, T.S. Theodore, P.L. Earl, B. Moss, D.J. Capon, M.A. Martin, In vitro mutagenesis identifies a region within the envelope gene of the human immunodeficiency virus that is critical for infectivity, J. Virol. 62 (1988) 139–147.
[23] H. Sakai, R. Shibata, J. Sakuragi, T. Kiyomasu, M. Kawamura, M. Hayami, A. Ishimoto, A. Adachi, Compatibility of rev gene activity in the four groups of primate lentiviruses, Virology 184 (1991) 513–520.
[24] R.A. Furuta, K. Sugiura, S. Kawakita, T. Inada, S. Ikehara, T. Matsuda, J. Fujisawa, Mouse model for the equilibration interaction between the host immune system and human T-cell leukemia virus type 1 gene expression, J. Virol. 76 (2002) 2703–2713.
[25] E. Bohnlein, M. Siekevitz, D.W. Ballard, J.W. Lowenthal, L. Rimsky, H. Bogerd, J. Hoffman, Y. Wano, B.R. Franza, W.C. Greene, Stimulation of the human immunodeficiency virus type 1 enhancer by the human T-cell leukemia virus type I tax gene product involves the action of inducible cellular proteins, J. Virol. 63 (1989) 1578–1586.
[26] H. Cheng, J. Tarnok, W.P. Parks, Human immunodeficiency virus type 1 genome activation induced by human T-cell leukemia virus type 1 Tax protein is through cooperation of NF-kappaB and Tat, J. Virol. 72 (1998) 6911–6916.
[27] T. Murakami, S. Ablan, E.O. Freed, Y. Tanaka, Regulation of human immunodeficiency virus type 1 Env-mediated membrane fusion by viral protease activity, J. Virol. 78 (2004) 1026–1031.
[28] S.C. Pettit, R. Sanchez, T. Smith, R. Wehbie, D. Derse, R. Swanstrom, HIV type 1 protease inhibitors fail to inhibit HTLV-I Gag processing in infected cells, AIDS Res. Hum. Retroviruses 14 (1998) 1007–1014.

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The '*O*-acyl isopeptide method' for the synthesis of difficult sequence-containing peptides: application to the synthesis of Alzheimer's disease-related amyloid β peptide (Aβ) 1–42

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Abstract: An efficient '*O*-acyl isopeptide method' for the synthesis of difficult sequence-containing peptides was applied successfully to the synthesis of amyloid β peptide (Aβ) 1–42 via a water-soluble *O*-acyl isopeptide of Aβ1–42, i.e. '26-*O*-acyl isoAβ1–42' (**6**). This paper describes the detailed synthesis of Aβ1–42 focusing on the importance of resin selection and the analysis of side reactions in the *O*-acyl isopeptide method. Protected '26-*O*-acyl isoAβ1–42' peptide resin was synthesized using 2-chlorotrityl chloride resin with minimum side reactions in comparison with other resins and deprotected crude 26-*O*-acyl isoAβ1–42 was easily purified by HPLC due to its relatively good purity and narrow elution with reasonable water solubility. This suggests that only one insertion of the isopeptide structure into the sequence of the 42-residue peptide can suppress the unfavourable nature of its difficult sequence. The migration of *O*-acyl isopeptide to intact Aβ1–42 under physiological conditions (pH 7.4) via *O–N* intramolecular acyl migration reaction was very rapid and no other by-product formation was observed while **6** was stable under storage conditions. These results concluded that our strategy not only overcomes the solubility problem in the synthesis of Aβ1–42 and can provide intact Aβ1–42 efficiently, but is also applicable in the synthesis of large difficult sequence-containing peptides at least up to 50 amino acids. This synthesis method would provide a biological evaluation system in Alzheimer's disease research, in which 26-*O*-acyl isoAβ1–42 can be stored in a solubilized form before use and then rapidly produces intact Aβ1–42 *in situ* during biological experiments. Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: Aβ1–42; *O*-acyl isopeptide method; Alzheimer's disease; Alzheimer's disease research tool; difficult sequence-containing peptide; *O–N* intramolecular acyl migration reaction

INTRODUCTION

The synthesis of 'difficult sequence'-containing peptides is one of the most problematic areas in peptide chemistry, and such peptides are often obtained with low yield and purity in solid-phase peptide synthesis (SPPS) [1–6]. These difficult sequences are generally hydrophobic and promote aggregation in solvents during synthesis and purification. This aggregation is attributed to intermolecular hydrophobic interaction and hydrogen bond network among resin-bound peptide chains, resulting in the formation of extended secondary structures such as β-sheets [1,2]. The tendency for aggregation depends on the nature of the peptide and side chain protecting groups. In particular, it is known that peptides with sequences containing Ala, Val, Ile, Asn and Gln residues in high frequency show a strong propensity for difficult sequences.

To solve the synthetic problem of difficult sequence-containing peptides, Sheppard and Johnson *et al.* reported a building block, 2-hydroxy-4-methoxybenzyl (Hmb), a protecting group for the backbone amide nitrogen [3,4]. Mutter *et al.* also introduced building blocks, so-called pseudo-prolines, which are dipeptide derivatives, consisting of Ser/Thr-derived oxazolidines or Cys-derived thiazolidine [5,6]. These special building blocks were designed to disrupt the secondary structure formed by interchain hydrogen bonding. However, to prepare building blocks, prior modifications of Fmoc-amino acids by 2–6 steps of additional solution phase synthesis are required in these approaches, and a strong acid treatment is also required to remove the building blocks. Therefore, the development of novel methods using conventional amino acid derivatives are of great significance in the synthesis of difficult sequence-containing peptides.

Hence, a novel and efficient '*O*-acyl isopeptide method' was developed for the synthesis of difficult sequence-containing peptides based on the synthesis of hydrophilic '*O*-acyl isopeptides' followed by *O–N* intramolecular acyl migration reaction (Figure 1A)

Abbreviations: PBS, phosphate buffered saline; Pmc, 2, 2, 5, 7, 8-pentamethylchroman-6-sulfonyl; Pns, phenylnorstatine = (2*R*, 3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid; otherwise as in *J. Peptide Sci* **9**: 1–8 (2003).

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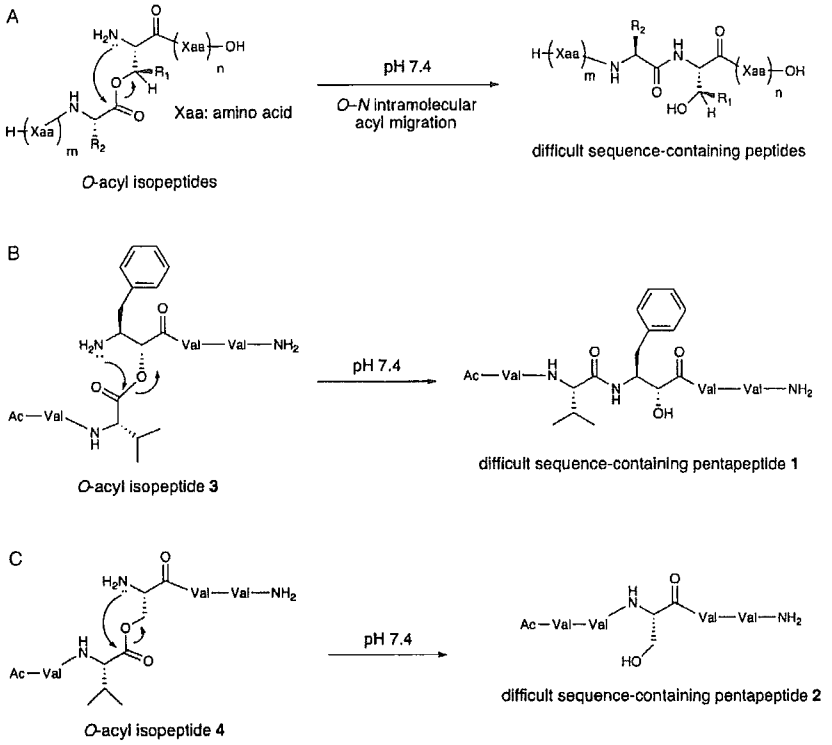


Figure 1 (A) '*O*-Acyl isopeptide method': the synthetic strategy for difficult sequence-containing peptides via the *O–N* intramolecular acyl migration reaction of *O*-acyl isopeptide, (B) application of the *O*-acyl isopeptide method for the synthesis of pentapeptide **1**, and (C) application of the *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_2 (**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_2 (**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_2O- 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 β peptide (Aβ) 1–42.

Amyloid β peptides (Aβs) 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 Aβ, which has been found to be neurotoxic *in vivo* and *in vitro* [26]. The predominant forms of Aβ mainly consist of 40- and 42-residue peptides (designated Aβ1–40 and Aβ1–42, respectively), which are proteolytically produced from amyloid precursor protein (APP) by enzymatic reactions [27]. Since Aβ1–42 is thought to play a more critical role in amyloid formation and the pathogenesis of AD than Aβ1–40, many studies using synthetic Aβ1–42 have been carried out to clarify the involvement of Aβ1–42 in AD [28–32].

However, Aβ1–42 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

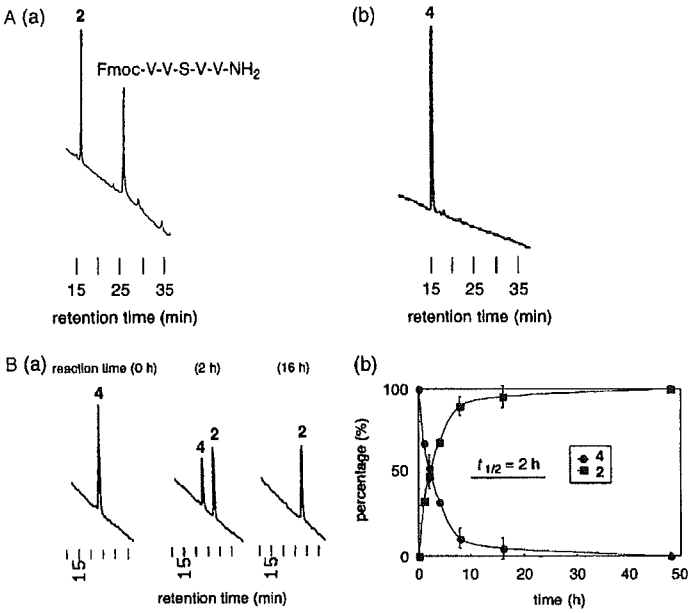


Figure 2 A: HPLC profiles of (a) crude peptide **2** and (b) its *O*-acyl isopeptide **4** (0–100% CH_3CN for 40 min, 230 nm). B: (a) periodical HPLC profiles (0–100% CH_3CN for 40 min, 230 nm) and (b) the graph for conversion of *O*-acyl isopeptide **4** to **2** via the *O*–*N* intramolecular acyl migration in phosphate buffered saline (pH 7.4, 25 °C).

HPLC purification of synthesized Aβ1–42 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^{35} to suppress aggregation [44] were employed. For effective HPLC purification, this peptide has also been purified under basic conditions [36,41]. Moreover, Aβ1–42 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, Aβ1–42 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 Aβ1–42, the peptide needs to be predissolved in dilute base solution. Therefore, an '*in situ*' system that could prepare intact monomer Aβ1–42 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 Aβ1–42 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 Aβ1–42 via a novel water-soluble isopeptide of Aβ1–42, i.e. '26-*O*-acyl isoAβ1–42 (26-AIAβ42, **6**)' (Figure 3) [47,48]. This overcomes the problems in the synthesis and storage of Aβ1–42. Although there are two Ser residues in Aβ1–42 at positions 8 and 26 with the capability of *O*–*N* intramolecular acyl migration, Ser^{26} was selected for *O*-acylation, since the adjacent Gly^{25} does not epimerize during ester bond formation (Figure 3). A previous rapid communication [47] reported the efficient synthesis of Aβ1–42 by the '*O*-acyl isopeptide method'. This paper describes the detailed synthesis of Aβ1–42, 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

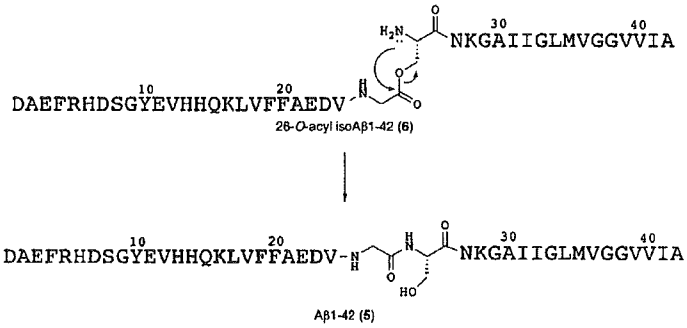


Figure 3 '*O*-Acyl isopeptide method' for the synthesis of Aβ1–42 (**5**): The production of Aβ1–42 (**5**) via the *O*–*N* intramolecular acyl migration of 26-*O*-acyl isoAβ1–42 (**6**).

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 α-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 × 150 mm; YMC Pack ODS AM302) with a binary solvent system: a linear gradient of CH_3CN in 0.1% aqueous TFA at a flow rate of 0.9 ml min^{-1} (temperature: 40 °C), detected at 230 nm. Preparative HPLC was carried out on a C18 reverse phase column (20 × 250 mm; YMC Pack ODS SH343-5) with a binary solvent system: a linear gradient of CH_3CN in 0.1% aqueous TFA at a flow rate of 5.0 ml min^{-1} (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: *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 *Nα*-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 (×5) and the completeness of each coupling was verified by the Kaiser test. *Nα*-Fmoc deprotection was carried out by treatment with piperidine (20% v/v in DMF) (2 ml, 1 min × 1 and 20 min × 1), followed by washing with DMF (1.5 ml, ×10) and chloroform (1.5 ml, ×5). If necessary, the coupling and deprotection cycles were repeated. After the peptide-resins were washed with methanol (1.5 ml, ×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 (×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_3CN 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$_2$ (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, ×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 µl, 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 µl, 0.222 mmol) in the presence of TEA (20.7 µl, 0.148 mmol) for 2 h. The peptide was cleaved from the resin using TFA (5 ml) in the presence of thioanisole (136.3 µl), *m*-cresol (136.3 µl) and distilled water (136.3 µl) 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 µm filter unit, and immediately injected into preparative HPLC with a 0.1% aqueous TFA–CH_3CN 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 $C_{32}H_{53}N_6O_7(M+H)^+$: 633.3976, found: 633.3982; HPLC analysis at 230 nm: purity was higher than 98%.

Ac-Val-Val-Pns-Val-Val-NH$_2$ (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 µl, 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 α-hydroxy group of Pns was performed using the DIPCDI (59.2 µl, 0.378 mmol)–DMAP (3.1 mg, 0.0252 mmol) method in CH_2Cl_2 (1.5 ml) for 16 h (×2), followed by the coupling of another Val residue, *N*-acetylation using Ac_2O (14.3 µl, 0.15 mmol)–TEA (17.5 µl, 0.126 mmol), TFA (4.4 ml)-thioanisole (117.2 µl)–*m*-cresol (117.2 µl)–distilled water (117.2 µl) 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 µl), filtered using a 0.46 µm filter unit, and immediately injected into preparative HPLC with a 0.1% aqueous TFA–CH_3CN 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 $C_{32}H_{53}N_6O_7(M+H)^+$: 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^{-1}) 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 $C_{32}H_{53}N_6O_7(M+H)^+$: 633.3976, found: 633.3983; HPLC analysis at 230 nm: purity was higher than 99%.

Ac-Val-Val-Ser-Val-Val-NH$_2$ (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 $C_{25}H_{47}N_6O_7(M+H)^+$: 543.3506, found: 543.3510; HPLC analysis at 230 nm: purity was higher than 95%.

Ac-Val-Val-Ser-Val-Val-NH$_2$ (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 $C_{25}H_{47}N_6O_7(M+H)^+$: 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 $C_{32}H_{53}N_6O_7(M+H)^+$: 543.3506, found: 543.3499; HPLC analysis at 230 nm: purity was higher than 96%.

Amyloid β peptide Aβ1-42 (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 DIPEA (26.2 µl, 0.15 mmol) in 1,2-dichloroethane (1.5 ml). After washing with DMF (1.5 ml, ×5), capping was performed with MeOH (200 µl) in the presence of DIPEA (52.5 µl, 0.3 mmol) in DMF for 20 min. After washing with DMF (×5), DMF–H_2O (1 : 1, ×5), $CHCl_3$ (×2) and MeOH (×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 µl, 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 µl)–thioanisole (64.5 µl)–H_2O (64.5 µl) 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_2O (1 ml) in the presence of NH_4I (12 mg, 0.08 mmol) and dimethylsulfide (6 µl, 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 µm filter unit, applied to preparative HPLC, and eluted using 0.1% aqueous TFA–CH_3CN. 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_{calc}: 4514.04; $M+H_{found}$: 4515.48; HPLC analysis at 230 nm: purity was >94%; the retention time on HPLC (0–100% CH_3CN for 40 min, 230 nm) of synthesized **5** was identical to that of commercially available Aβ1–42 (Peptide Institute, Inc., Osaka, Japan).

Amyloid β peptide Aβ1-42 (5, in O-acyl isopeptide method). After protected Aβ27-42-resin (chlorotrityl chloride resin, 0.1 mmol) was synthesized in the same manner as described in the synthesis of Aβ1–42 in the conventional method, Boc-Ser-OH (48.1 mg, 0.25 mmol) was coupled by the DIPCDI (36.4 µl, 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 µl, 0.3 mmol)–DMAP (2.3 mg, 0.02 mmol) method in CH_2Cl_2 (1.5 ml) for 16 h (×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 µl)–thioanisole (88.3 µl)–H_2O (88.3 µl) 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_2O (2 ml) in the presence of NH_4I (21.8 mg, 0.17 mmol) and dimethylsulfide (11 µl, 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 µm filter unit, applied to preparative HPLC, and eluted using a 0.1% aqueous TFA–CH_3CN. 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_{calc}: 4514.04; $M+H_{found}$: 4515.26; HPLC analysis at 230 nm: purity was >96%.

The purified **6** was dissolved in H_2O and stirred for 48 h at room temperature followed by lyophilization to yield **5** quantitatively as a TFA salt. MALDI-MS (TOF): M_{calc}: 4514.04; $M+H_{found}$: 4515.48; HPLC analysis at 230 nm: purity was >95%; the retention time on HPLC (0–100% CH_3CN for 40 min, 230 nm) of synthesized **5** was identical to that of commercially available Aβ1–42 (Peptide Institute, Inc., Osaka, Japan).

26-O-Ser-Aβ26-42 (15). After protected Aβ26–42-resin (**9**, chlorotrityl 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 µl, 0.138 mmol) and DMAP (1.3 mg, 0.0092 mmol) in CH_2Cl_2 (1.5 ml) for 16 h (×2). The peptide-resin was treated with 20% piperidine–DMF for 20 min and TFA (1.79 ml)–*m*-cresol (45.4 µl)–thioanisole (45.4 µl)–H_2O (45.4 µl) 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_2O (0.5 ml) in the presence of NH_4I (5.45 mg, 0.043 mmol) and dimethylsulfide (11 µl, 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 μm filter unit, applied to preparative HPLC, and eluted using 0.1% aqueous TFA–CH_3CN. The desired fractions were collected and immediately lyophilized to afford the titled peptide **15** as a white amorphous powder. MALDI-MS (TOF): M_{calc}: 1686.03; $M + H_{found}$: 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 μm or 0.46 μm filter unit, Ultrafree®-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 μl of PBS (pH 7.4) were added 4 μl of DMSO and 1 μl 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 μl of DMSO was added to the samples and 500 μl of the mixture was directly analysed by RP-HPLC. HPLC was performed using a C18 (4.6 × 150 mm; YMC Pack ODS AM302) reverse-phase column with a binary solvent system: linear gradient of CH_3CN (40%–100%, 30 min) in 0.1% aqueous TFA at a flow rate of 0.9 ml min^{-1}, detected at UV 230 nm.

Stability of *O*-Acyl IsoAβ1-42 (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 μl of buffered saline (pH 7.4: PBS, pH 4.9: PBS, pH 3.5: acetate buffer) was added 3–6 μl 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 μl of hexafluoroisopropanol was added to the samples and 500 μl of the mixture was directly analysed by RP-HPLC.

RESULTS AND DISCUSSION

In the synthesis of 26-AIAβ42 (**6**) based on the *O*-acyl isopeptide method (Scheme 1), Fmoc-Ala-*O*-chlorotrityl 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 β-hydroxy group of Ser using the DIPCDI–DMAP method in CH_2Cl_2 to obtain ester **10**. 26-AIAβ42-resin (**13**) was obtained through the further coupling of additional amino acid residues using the conventional manner. Finally, 26-AIAβ42 (**6**) was obtained as a major product (Figure 4A) by treatment with TFA–*m*-cresol–thioanisole–H_2O (92.5 : 2.5 : 2.5 : 2.5) for 90 min

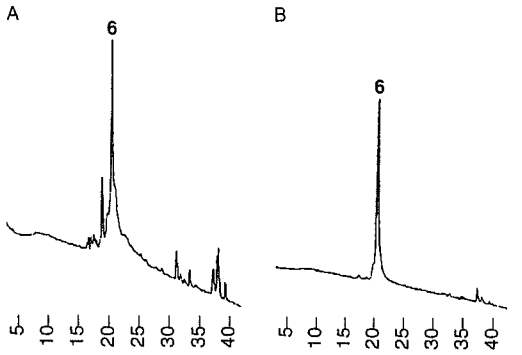


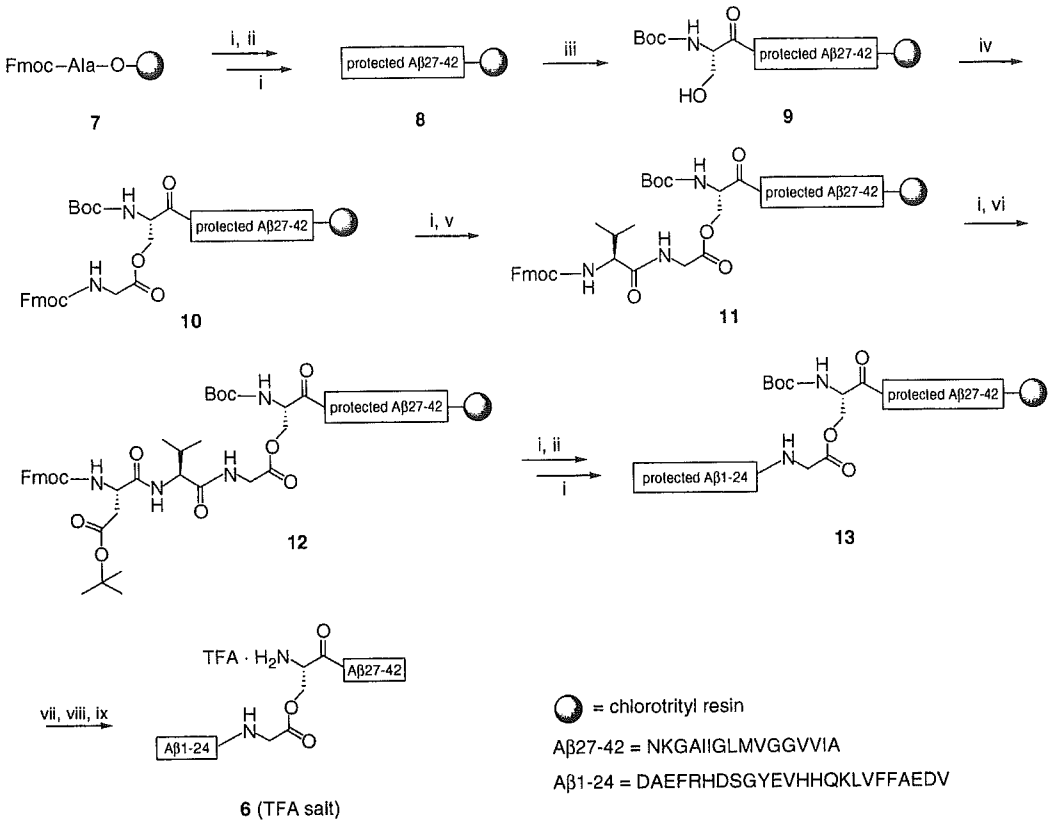
Figure 4 HPLC profiles of (A) crude and (B) purified 26-AIAβ42 (**6**). Analytical HPLC was performed using a C18 reverse-phase column (4.6 × 150 mm; YMC Pack ODS AM302) with a binary solvent system: the linear gradient of CH_3CN (0–100% CH_3CN for 40 min) in 0.1% aqueous TFA at a flow rate of 0.9 ml min^{-1} (temperature: 40 °C), detected at 230 nm.

followed by reduction with NH_4I–dimethylsulfide for 60 min in TFA : H_2O (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 β-hydroxy group of Ser^{26}, 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 β-hydroxy group of Ser^{26} 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 isoAβ25–42 by HPLC analysis (data not shown) [TOF-MS: M_{calc}: 1878.24; $M + H_{found}$: 1879.29], and Aβ26–42, 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), Aβ1–25 (DAEFRHDSGYEVHHQKLVFFAEDVG) was not detected as a by-product, although a very small amount (1.6%, HPLC yield) of Aβ26–42 (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 Aβ26–42 formation in crude **6** might be



Scheme 1 Reagents/conditions: (i) 20% piperidine/DMF, 20 min; (ii) Fmoc-AA-OH (2.5 eq), DIPCDI (2.5 eq), HOBt (2.5 eq), DMF, 2 h; (iii) Boc-Ser-OH (2.5 eq), DIPCDI (2.5 eq), HOBt (2.5 eq), DMF, 2 h; (iv) Fmoc-Gly-OH (3.0 eq), DIPCDI (3.0 eq), DMAP (0.2 eq), CH_2Cl_2, 16 h × 2; (v) Fmoc-Val-OH (2.5 eq), DIPCDI (2.5 eq), HOBt (2.5 eq), DMF, 2 h; (vi) Fmoc-Asp(O*t*Bu)-OH (2.5 eq), DIPCDI (2.5 eq), HOBt (2.5 eq), DMF, 2 h; (vii) TFA–*m*-cresol–thioanisole–H_2O (92.5 : 2.5 : 2.5 : 2.5), 90 min; (viii) NH_4I (20 eq), dimethylsulfide (20 eq), TFA : H_2O (2 : 1), 60 min, 0 °C; (ix) preparative HPLC (the linear gradient of CH_3CN 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_2 (**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^{25} 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_3CN. 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®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_{calc} : 2833.11 M + H_{found} : 2934.11, Aβ26–42, M_{calc} : 1598.95; M + H_{found} : 1599.28) in the resultant crude **6**, indicating that

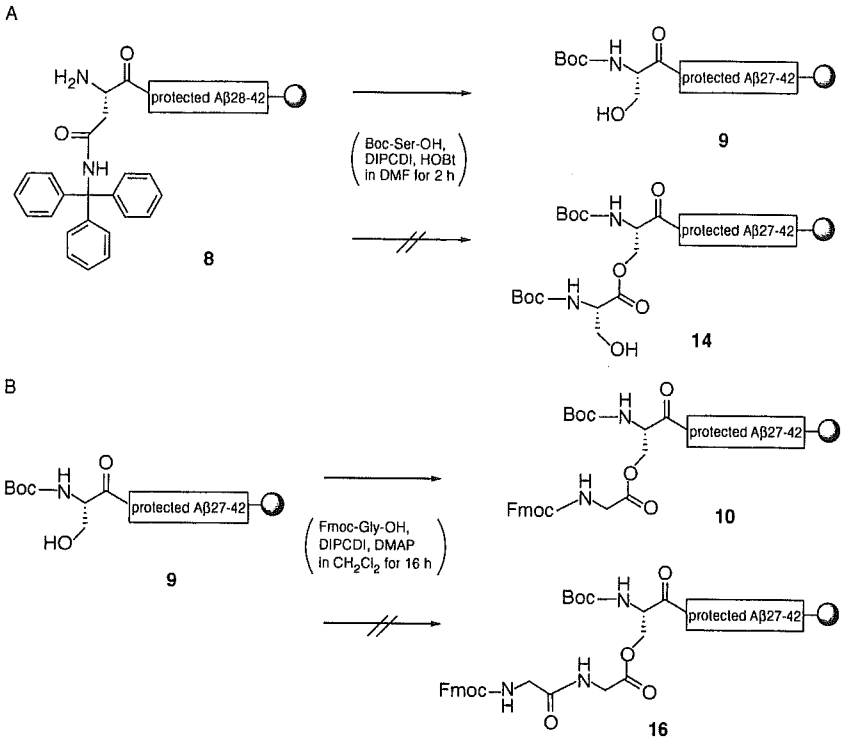


Figure 5 Examination whether a side reaction occurred in the reaction of (A) Boc-Ser-OH insertion to **8** (Scheme 1, step iii) and (B) esterification of **9** (Scheme 1, step iv).

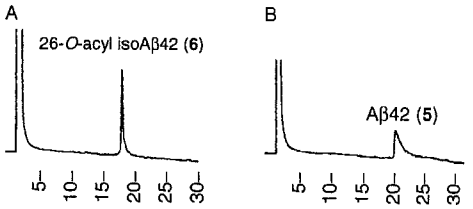


Figure 6 HPLC profiles of pure (A) 26-AIAβ42 (**6**) and (B) Aβ1–42 (**5**). The same quantity (2.3 nmol) of both peptides was applied to the HPLC column as DMSO solution. Analytical HPLC was performed using a C18 reverse-phase column (4.6 × 150 mm; YMC Pack ODS AM302) with a binary solvent system: the linear gradient of CH_3CN (25%–55% CH_3CN for 60 min) in 0.1% aqueous TFA at a flow rate of 0.9 ml min^{-1} (temperature: 40 °C), detected at 230 nm.

the ester bond between Gly^{25} and Ser^{26} was significantly cleaved by the treatment of TFA–*m*-cresol–thioanisole–H_2O (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^{-1}, 100-fold higher than that of Aβ1–42 (**5**, 0.14 mg ml^{-1}). 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_3CN, 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 Aβ1–42 was suppressed by only one insertion of the isopeptide structure. Recent solution-state NMR studies of the Aβ1–40 and Aβ1–42 by Zagorski *et al.* [32] indicated that the Ser^{26} residue comprises turn- or bendlike structures that bring two β-sheets in contact and hydrogen bonding among peptide chains, which is associated with β-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 Aβ1–42 (**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^{25} 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 Aβ1–42, *in situ* to investigate the inherent biological function of Aβ1–42 in AD. The conversion of **6** (TFA salt) in water for 48 h at room temperature followed by lyophilization yielded Aβ1–42 (**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 Aβ1–42 as a general method. In addition, rapid migration of *O*-acyl isopeptides to intact Aβ1–42 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 Aβ1–42, but also provides a novel tool for the biological evaluation system in AD research, in which 26-*O*-acyl isoAβ1–42 can be stored in a solubilized form before use and rapidly produces intact monomer Aβ1–42 *in situ* during biological experiments.

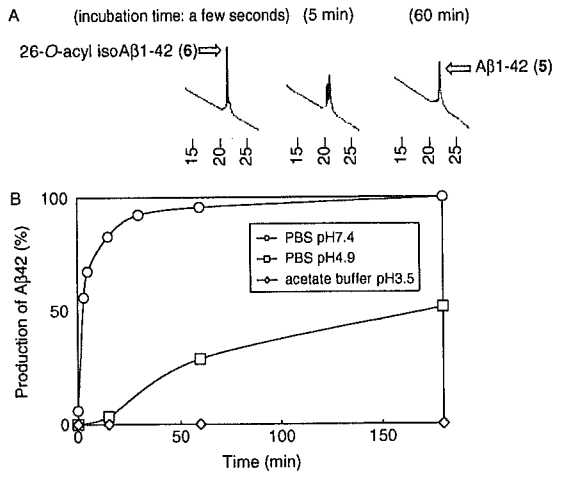


Figure 7 (A) HPLC profiles of the conversion of 26-AIAβ42 (**6**) to Aβ1–42 (**5**) via *O–N* intramolecular acyl migration in PBS (pH 7.4) at 25 °C and (B) a graph of the production of Aβ1–42 (**5**) in various pH conditions at 25 °C. Analytical HPLC was performed using a C18 reverse-phase column (4.6 × 150 mm; YMC Pack ODS AM302) with a binary solvent system: the linear gradient of CH_3CN (0–100% CH_3CN for 40 min) in 0.1% aqueous TFA at a flow rate of 0.9 ml min^{-1} (temperature: 40 °C), detected at 230 nm.

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REFERENCES

1. Kent SBH. Chemical synthesis of peptides and proteins. *Annu. Rev. Biochem.* 1988; **57**: 957–990.
2. Tam JP, Lu YA. Coupling difficulty associated with interchain clustering and phase transition in solid phase peptide synthesis. *J. Am. Chem. Soc.* 1995; **117**: 12058–12063.
3. Johnson T, Quibell M, Owen D, Sheppard RC. A reversible protecting group for the amide bond in peptides. Use in the synthesis of 'difficult sequences'. *J. Chem. Soc. Chem. Commun.* 1993; 369–372.
4. For a review, see: Sheppard R. The fluorenylmethoxycarbonyl group in solid phase synthesis. *J. Peptide Sci.* 2003; **9**: 545–552.
5. Wöhr T, Wahl F, Nefzi A, Rohwedder B, Sato T, Sun X, Mutter M. Pseudo-prolines as a solubilizing, structure-disrupting protection technique in peptide synthesis. *J. Am. Chem. Soc.* 1996; **118**: 9218–9227.
6. Guichou J-F, Patiny L, Mutter M. Pseudo-prolines (ψPro): Direct insertion of ψPro systems into cysteine containing peptides. *Tetrahedron Lett.* 2002; **43**: 4389–4390.
7. Sohma Y, Sasaki M, Ziora Z, Takahashi N, Kimura T, Hayashi Y, Kiso Y. A novel approach for the hydrophobic peptides synthesis and purification through *O–N* intramolecular acyl migration reaction. *Peptides, Peptide Revolution: Genomics, Proteomics & Therapeutics*. Kluwer Academic: Netherlands, 2003; 67–68.
8. Sohma Y, Sasaki M, Hayashi Y, Kimura T, Kiso Y. Novel and efficient synthesis of difficult sequence-containing peptides through *O–N* intramolecular acyl migration reaction of *O*-acyl isopeptides. *Chem. Commun.* 2004; 124–125.
9. Stewart JM. Protection of the hydroxyl group in peptide synthesis. In *The Peptides*, Gross E. Meienhofer J (eds). Academic Press: NY, 1981; **3**: 169–201.
10. Tamamura H, Kato T, Otaka A, Fujii N. Synthesis of potent β-secretase inhibitors containing a hydroxyethylamine dipeptide

isostere and their structure-activity relationship studies. *Org. Biomol. Chem.* 2003; **1**: 2468–2473.
11. Mouls L, Subra G, Enjalbal C, Martinez J, Aubagnac J-L. O-N-Acyl migration in *N*-terminal serine-containing peptides: mass spectrometric elucidation and subsequent development of site-directed acylation protocols. *Terahedron Lett.* 2004; **45**: 1173–1178.
12. Nishizawa R, Saino T, Takita T, Suda H, Aoyagi T, Umezawa H. Synthesis and structure-activity relations of bestatin analogs, inhibitors of aminopeptidase B. *J. Med. Chem.* 1977; **20**: 510–515.
13. Iizuka K, Kamijo T, Harada H, Akahane K, Kubota T, Umeyama H, Ishida T, Kiso Y. Orally potent human renin inhibitors derived from angiotensinogen transition state: design, synthesis, and mode of interaction. *J. Med. Chem.* 1990; **33**: 2707–2714.
14. Mimoto T, Imai J, Tanaka S, Hattori N, Takahashi O, Kisanuki S, Nagano Y, Shintani M, Hayashi H, Sakikawa H, Akaji K, Kiso Y. Rational design and synthesis of a novel class of active site-targeted HIV protease inhibitors containing a hydroxymethylcarbonyl isostere. Use of phenylnorstatine or allophenylnorstatine as a transition-state mimic. *Chem. Pharm. Bull.* 1991; **39**: 2465–2467.
15. Nezami A, Luque I, Kimura T, Kiso Y, Freire E. Identification and characterization of allophenylnorstatine-based inhibitors of plasmepsin II, an antimalarial target. *Biochemistry* 2002; **41**: 2273–2280.
16. Kimura T, Shuto D, Hamada Y, Igawa N, Kasai S, Liu P, Hidaka K, Hamada T, Hayashi Y, Kiso Y. Design and synthesis of highly active Alzheimer's β-secretase (BACE1) inhibitors, KMI-420 and KMI-429, with enhanced chemical stability. *Bioorg. Med. Chem. Lett.* 2005; **15**: 211–215.
17. Kimura T, Ohtake J, Nakata S, Enomoto H, Moriwaki H, Akaji K, Kiso Y. Synthesis of prodrugs of HIV protease inhibitors. *Peptide Chemistry 1994*, Ohno M. (ed.) Protein Research Foundation: Osaka, 1995; 157–160.
18. Oliyai R, Stella VJ. Structural factors affecting the kinetics of O,N-acyl transfer in potential O-peptide prodrugs. *Bioorg. Med. Chem. Lett.* 1995; **5**: 2735–2740.
19. Kiso Y, Kimura T, Ohtake J, Nakata S, Enomoto H, Moriwaki H, Nakatani M, Akaji K. 'O → N intramolecular acyl migration' - type prodrugs of tripeptide inhibitors of HIV protease. *Peptides: Chemistry, Structure and Biology*, Mayflower Scientific: England, 1996; 157–159.
20. Hamada Y, Ohtake J, Sohma Y, Kimura T, Hayashi Y, Kiso Y. New water-soluble prodrugs of HIV protease inhibitors based on O → N intramolecular acyl migration. *Bioorg. Med. Chem.* 2002; **10**: 4155–4167.
21. Hamada Y, Matsumoto H, Kimura T, Hayashi Y, Kiso Y. Effect of the acyl groups on O → N acyl migration in the water-soluble prodrugs of HIV-1 protease inhibitor. *Bioorg. Med. Chem. Lett.* 2003; **13**: 2727–2730.
22. Hayashi Y, Skwarczynski M, Hamada Y, Sohma Y, Kimura T, Kiso Y. A novel approach of water-soluble paclitaxel prodrug with no auxiliary and no byproduct: design and synthesis of isotaxel. *J. Med. Chem.* 2003; **46**: 3782–3784.
23. Skwarczynski M, Sohma Y, Kimura M, Hayashi Y, Kimura T, Kiso Y. O–N Intramolecular acyl migration strategy in water-soluble prodrugs of taxoids. *Bioorg. Med. Chem. Lett.* 2003; **13**: 4441–4444.
24. Hamada Y, Matsumoto H, Yamaguchi S, Kimura T, Hayashi Y, Kiso Y. Water-soluble prodrugs of dipeptide HIV protease inhibitors based on O → N intramolecular acyl migration: Design, synthesis and kinetic study. *Bioorg. Med. Chem.* 2004; **12**: 159–170.
25. Selkoe DJ. Translating cell biology into therapeutic advances in Alzheimer's disease. *Nature* 1999; **399**: A23–A31.
26. Geula C, Wu CK, Saroff D, Lorenzo A, Yuan ML, Yankner BA. Aging renders the brain vulnerable to amyloid β-protein neurotoxicity. *Nature Med.* 1998; **4**: 827–831.
27. Sinha S, Lieberburg I. Cellular mechanisms of beta-amyloid production and secretion. *Proc. Natl Acad. Sci. USA* 1999; **96**: 11 049–11 053.
28. Iwatsubo T, Odaka A, Suzuki N, Mizusawa H, Nukina N, Ihara Y. Visualization of Aβ42(43) and Aβ40 in senile plaques with end-specific Aβ monoclonals: evidence that an initially deposited species is Aβ42(43). *Neuron* 1994; **13**: 45–53.
29. Younkin SG. Evidence that Aβ42 is the real culprit in Alzheimer's disease. *Ann. Neurol.* 1995; **37**: 287–288.
30. Dahlgren KN, Manelli AM, Stine WB Jr, Baker LK, Krafft GA, LaDu MJ. Oligomeric and fibrillar species of amyloid-β peptides differentially affect neuronal viability. *J. Biol. Chem.* 2002; **277**: 32 046–32 053.
31. Bitan G, Tarus B, Vollers SS, Lashuel HA, Condron MM, Straub JE, Teplow DB. A molecular switch in amyloid assembly: Met35 and amyloid β-protein oligomerization. *J. Am. Chem. Soc.* 2003; **125**: 15 359–15 365.
32. Hou L, Shao H, Zhang Y, Li H, Menon NK, Neuhaus EB, Brewer JM, Byeon I-J L, Ray DG, Vitek MP, Iwashita T, Makula RA, Przybyla AB, Zagorski MG. Solution NMR studies of the Aβ(1–40) and Aβ(1–42) peptides establish that the Met35 oxidation state affects the mechanism of amyloid formation. *J. Am. Chem. Soc.* 2004; **126**: 1992–2005.
33. Burdick D, Soreghan B, Kwon M, Kosmoski J, Knauer M, Henschen A, Yates J, Cotman C, Glabe C. Assembly and aggregation properties of synthetic Alzheimer's A4/β amyloid peptide analogs. *J. Biol. Chem.* 1992; **267**: 546–554.
34. Quibell M, Turnell WG, Johnson T. Improved preparation of β-amyloid(1–43): structural insight leading to optimized positioning of N-(2-hydroxy-4-methoxybenzyl) (Hmb) backbone amide protection. *J. Chem. Soc., Perkin Trans. 1* 1995; 2019–2024.
35. Shen C-L, Murphy RM. Solvent effects on self-assembly of β-amyloid peptide. *Biophys. J.* 1995; **69**: 640–651.
36. Fukuda H, Shimizu T, Nakajima M, Mori H, Shirasawa T. Synthesis, aggregation, and neurotoxicity of the Alzheimer's Aβ1–42 amyloid peptide and its isoaspartyl isomers. *Bioorg. Med. Chem. Lett.* 1999; **9**: 953–956.
37. Milton SCF, Milton RCD, Kates SA, Glabe C. Improved synthesis and purification of Alzheimer's Aβ1–42 and analogs. *Lett. Peptide Sci.* 1999; **6**: 151–156.
38. El-Agnaf OMA, Mahil DS, Patel BP, Austen BM. Oligomerization and toxicity of β-amyloid-42 implicated in Alzheimer's disease. *Biochem. Biophys. Res. Commun.* 2000; **273**: 1003–1007.
39. Tickler AK, Barrow CJ, Wade JD. Improved preparation of amyloid-β peptides using DBU as N^{α}-Fmoc deprotection reagent. *J. Peptide Sci.* 2001; **7**: 488–494.
40. Inui T, Bódi J, Nishio H, Nishiuchi Y, Kimura T. Synthesis of amyloid β-peptides in solution employing chloroform-phenol mixed solvent for facile segment condensation of sparingly soluble protected peptides. *Lett. Peptide Sci.* 2002; **8**: 319–330.
41. Murakami K, Irie K, Morimoto A, Ohigashi H, Shindo M, Nagao M, Shimizu T, Shirasawa T. Neurotoxicity and physicochemical properties of Aβ mutant peptides from cerebral amyloid angiopathy: implication for the pathogenesis of cerebral amyloid angiopathy and Alzheimer's disease. *J. Biol. Chem.* 2003; **278**: 46 179–46 187.
42. Tickler AK, Clippingdale AB, Wade JD. Amyloid-β as a 'difficult sequence' in solid phase peptide synthesis. *Protein Peptide Lett.* 2004; **11**: 377–384.
43. D'Ursi AM, Armenante MR, Guerrini R, Salvadori S, Sorrentino G, Picone D. Solution structure of amyloid β-peptide (25–35) in different media. *J. Med. Chem.* 2004; **47**: 4231–4238.
44. Kim YS, Moss JA, Janda KD. Biological tuning of synthetic tactics in solid-phase synthesis: Application to Aβ(1–42). *J. Org. Chem.* 2004; **69**: 7776–7778.
45. Stine WB Jr, Dahlgren KN, Krafft GA, Ladu MJ. *In vitro* characterization of conditions for amyloid-β peptide oligomerization and fibrillogenesis. *J. Biol. Chem.* 2003; **278**: 11 612–11 622.
46. Soto C, Castano EM, Kumar RA, Beavis RC, Frangione B. Fibrillogenesis of synthetic amyloid-beta peptides is dependent on their initial secondary structure. *Neurosci. Lett.* 1995; **200**: 105–108.

47. Sohma Y, Sasaki M, Hayashi Y, Kimura T, Kiso Y. Design and synthesis of a novel water-soluble Aβ1–42 isopeptide: an efficient strategy for the preparation of Alzheimer's disease-related peptide, Aβ1–42, via O–N intramolecular acyl migration reaction. *Tetrahedron Lett.* 2004; **45**: 5965–5968.
48. Sohma Y, Hayashi Y, Skwarczynski M, Hamada Y, Sasaki M, Kimura T, Kiso Y. O–N Intramolecular acyl migration reaction in the development of prodrugs and the synthesis of difficult sequence-containing bioactive peptides. *Biopolymers* 2004; **76**: 344–356.
49. Bayer E. Protein synthesis. *Angew. Chem. Int. Ed.* 1991; **30**: 113–129.
50. Sheppard RC, Williams BJ. Acid-labile resin linkage agents for use in solid phase peptide synthesis. *Int. J. Peptide Protein Res.* 1982; **20**: 451–454.
51. Carpino LA, Krause E, Sferdean CD, Schuemann M, Fabian H, Bienert M, Beyermann M. Synthesis of 'difficult' peptide sequences: application of a depsipeptide technique to the Jung-Redemann 10- and 26-mers and the amyloid peptide Aβ(1–42). *Tetrahedron Lett.* 2004; **45**: 7519–7523.
52. Mutter M, Chandravarkar A, Boyat C, Lopez J, Santos SD, Mandal B, Mimna R, Murat K, Patiny L, Saucede L, Tuchscherer G. Switch peptides in statu nascendi: induction of conformational transitions relevant to degenerative diseases. *Angew. Chem. Int. Ed.* 2004; **43**: 4172–4178.

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'*O*-Acyl isopeptide method' for the efficient preparation of amyloid β peptide 1–42 mutants

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Abstract—Novel water-soluble isopeptides of Aβ1–42 mutants, '26-*O*-acyl isoAβ1–42 (26-AIAβ42) mutants', which were efficiently converted to intact Aβ1–42 mutants with no byproduct formation under physiological conditions, were synthesized. These isopeptides provide a new system useful for investigating the biological function of Aβ1–42 mutants.

1. Introduction

Amyloid β peptides (Aβs) are the main proteinaceous component of amyloid plaques found in the brain as a pathognomonic feature of Alzheimer's disease (AD),[1] and have been found to be neurotoxic in vivo and in vitro.[2] Although the predominant forms of Aβ mainly consist of 40- and 42-residue peptides, designated Aβ1–40 and Aβ1–42, respectively, Aβ1–42 is thought to play a more critical role in amyloid formation and the pathogenesis of AD than Aβ1–40.[3] In addition, not only wild-type Aβ1–42 (D^{1}AEFRHDSGY^{10}EVHHQKLVF-F^{20}AEDVGSNKGA^{30}IIGLMVGGVV^{40}IA) observed in AD, but missense mutations inside the Aβ-coding region in the amyloid precursor protein (APP) gene are also well-known. These mutations, known as Flemish-(A21G),[4] Arctic-(E22G),[5] Dutch-(E22Q),[6] Italian-(E22K),[7] and Iowa-type (D23N)[8] are found at positions 21–23 of Aβ. 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.[9] Recent studies have discussed several differences among Aβ mutants in amyloid formation, metabolism, and elimination, which are related to the progression of AD-like diseases.[10] Hence, more detailed studies comparing these features among Aβ mutants would afford crucial information for understanding the mechanism of the diseases. A sufficient supply of synthetic Aβ mutants would be key to this research.

Numerous studies have supported the hypothesis that neurotoxicity and the kinetics of Aβ1–42 aggregation are directly related to the assembly state in solution. However, the pathological self-assembly of Aβ1–42 in amyloid plaque formation, a currently unexplained process, is very difficult to demonstrate in vitro due to its uncontrolled polymerization. For example, synthesized Aβ1–42 already contains variable oligomeric forms,[3d,11] as Aβ1–42 undergoes time- and concentration-dependent aggregation in an aqueous TFA–acetonitrile solution used in HPLC purification.[12] Moreover, the Aβ1–42 monomer easily forms an aggregate even in a standard storage solution such as dimethylsulfoxide (DMSO).[13] Uncontrolled self-assembly in an in vitro experiment might cause considerable discrepancy in the biological data.[3d,11] Therefore, this highly agglutinative feature of Aβ1–42 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 Aβ1–42 in various media also results in synthetic difficulties with this peptide,[12,14] a so-called 'difficult sequence-containing peptide'.[15] In particular, in conventional reverse-phase HPLC purification of synthesized Aβ1–42 with the

Keywords: *O*-Acyl isopeptide method; Alzheimer's disease; Aβ1–42; Aβ1–42 mutant; 26-AIAβ42; *O–N* intramolecular acyl migration reaction.
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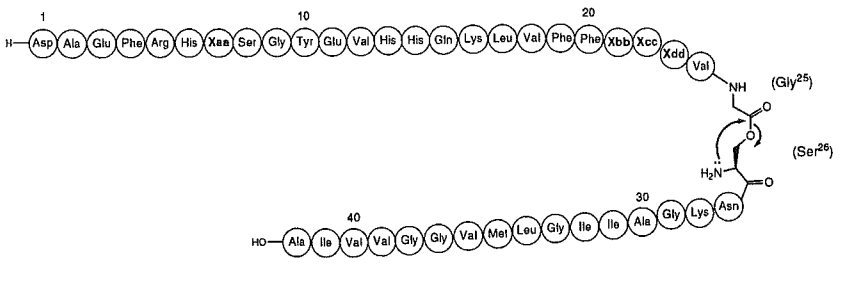
aqueous TFA–acetonitrile system, it is too laborious to remove impurities accumulated during solid-phase peptide synthesis (SPPS) due to its low solubility and broad elution under either acidic or neutral conditions.

An 'in situ' system that prepares an intact monomer Aβ1–42 under physiological experimental conditions while suppressing the spontaneous self-assembly of Aβ1–42 under storage conditions would be advantageous in understanding the inherent pathological functions of agglutinative Aβ1–42 in AD-like diseases. For this purpose, based on the '*O*-acyl isopeptide method',[16] we developed a novel water-soluble isopeptide of wild-type Aβ1–42, '26-*O*-acyl isoAβ1–42' (26-AIAβ42, **8**, Fig. 1).[17] This isopeptide exhibited higher water solubility than Aβ1–42 (**1**), and *O*–*N* intramolecular acyl migration reaction[18,19] 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 Aβ1–42 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 Aβ1–42 mutant under physiological conditions (Fig. 1) via *O*–*N* intramoleculor acyl migration, providing a useful new system for investigating the biological function of Aβ1–42 mutants.

2. Chemistry

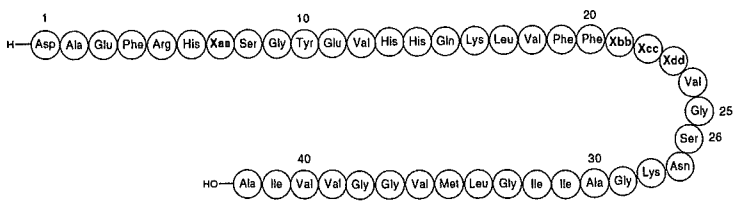
The synthetic scheme of Dutch-type 26-AIAβ42 (E22Q, **12**), based on the *O*-acyl isopeptide method, is depicted in Scheme 1 as a representative example. Fmoc-Ala-*O*-chlorotrityl resin (**15**) was employed according to our previous study.[17c] Fmoc-protected amino acids were sequentially coupled using 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



26-*O*-acyl isoAβ1–42 (**8**–**14**)

Entry	Xaa^{7}	Xbb^{21}	Xcc^{22}	Xdd^{23}
8 (wild)	Asp	Ala	Glu	Asp
9 (Japanese-Tottori)	Asn	Ala	Glu	Asp
10 (Flemish)	Asp	Gly	Glu	Asp
11 (Arctic)	Asp	Ala	Gly	Asp

Entry	Xaa^{7}	Xbb^{21}	Xcc^{22}	Xdd^{23}
12 (Dutch)	Asp	Ala	Gln	Asp
13 (Italian)	Asp	Ala	Lys	Asp
14 (Iowa)	Asp	Ala	Glu	Asn

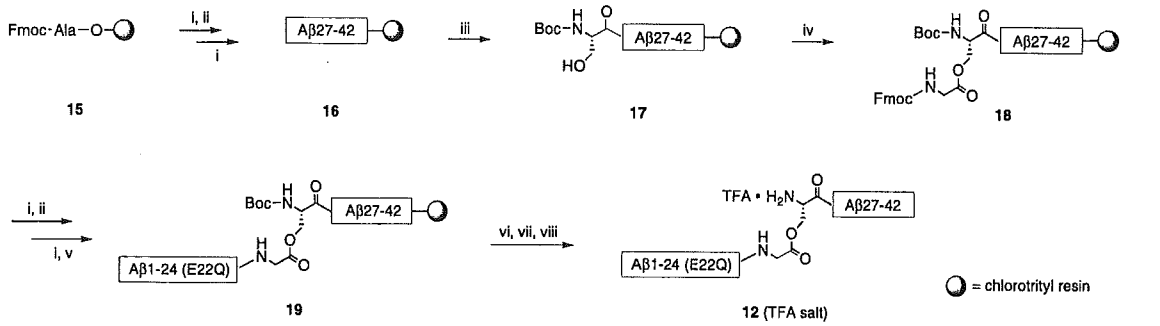


Aβ1–42 (1–7)

Entry	Xaa^{7}	Xbb^{21}	Xcc^{22}	Xdd^{23}
1 (wild)	Asp	Ala	Glu	Asp
2 (Japanese-Tottori)	Asn	Ala	Glu	Asp
3 (Flemish)	Asp	Gly	Glu	Asp
4 (Arctic)	Asp	Ala	Gly	Asp

Entry	Xaa^{7}	Xbb^{21}	Xcc^{22}	Xdd^{23}
5 (Dutch)	Asp	Ala	Gln	Asp
6 (Italian)	Asp	Ala	Lys	Asp
7 (Iowa)	Asp	Ala	Glu	Asn

Figure 1. '*O*-Acyl isopeptide method' for the efficient preparation of Aβ1–42 (**1**–**7**): the production of Aβ1–42 (**1**–**7**) via *O*–*N* intramolecular acyl migration of 26-*O*-acyl isoAβ1–42 (**8**–**14**).



Scheme 1. Reagents and conditions: (i) 20% piperidine/DMF, 20 min; (ii) Fmoc-AA-OH (2.5 equiv), DIPCDI (2.5 equiv), HOBt (2.5 equiv), DMF, 2 h; (iii) Boc-Ser-OH (2.5 equiv), DIPCDI (2.5 equiv), HOBt (2.5 equiv), DMF, 2 h; (iv) Fmoc-Gly-OH (15.0 equiv), DIPCDI (15.0 equiv), DMAP (0.3 equiv), CH_2Cl_2, 4 h × 2; (v) Boc-Asp (O*t*Bu)-OH (2.5 equiv), DIPCDI (2.5 equiv), HOBt (2.5 equiv), DMF, 2 h; (vi) TFA/*m*-cresol/thioanisole/H_2O (92.5:2.5:2.5:2.5), 90 min; (vii) NH_4I (20 equiv), dimethylsulfide (20 equiv), TFA/H_2O (2:1), 60 min, 0 °C; (viii) preparative HPLC (the linear gradient of CH_3CN in 0.1% aqueous TFA).

method (2 h) to obtain **17**, which was coupled with Fmoc-Gly-OH at the β-hydroxy group of Ser using the DIPCDI–DMAP method[18b] in CH_2Cl_2 to obtain ester **18**. A protected Dutch-type 26-AIAβ42 resin (**19**) was obtained through the subsequent coupling of amino acid residues using the conventional manner. Finally, Dutch-type 26-AIAβ42 (**12**) was obtained as a major product (Fig. 2A) by treating **19** with TFA/*m*-cresol/thioanisole/H_2O (92.5:2.5:2.5:2.5) for 90 min followed by reduction with NH_4I–dimethylsulfide for 60 min in TFA/H_2O (2:1). Other 26-AIAβ42 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 Aβ1–25 (DAEFRHDSG YEVHHQKLVFFAQDVG) was not detected as a by-product, although a small amount (1.8%, HPLC yield) of Aβ26–42 (SNKGAIIGLMVGGVVIA) was observed. This result indicates that the formed ester bond between Gly^{25} and Ser^{26} was almost stable in both 20% piperidine and TFA treatments, comparable to the synthesis of wild-type 26-AIAβ42 (**8**), in which 1.6% of Aβ26–42 was detected in a crude sample.[17] The slight formation of Aβ26–42 observed in crude **12** might be attributed to diketopiperazine formation during Fmoc group deprotection of Val^{24} 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).[17c] The crude HPLC profiles of other 26-AIAβ42 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–CH_3CN. 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

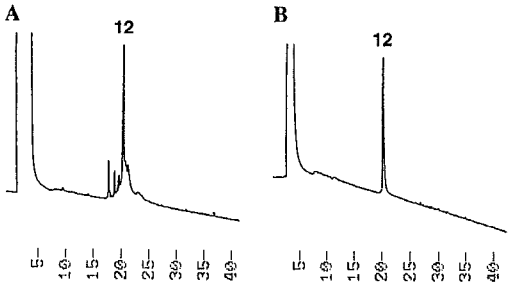


Figure 2. HPLC profiles of (A) crude and (B) purified Dutch-type 26-AIAβ42 (**12**). Analytical HPLC was performed using a C18 reverse-phase column (4.6 × 150 mm; YMC Pack ODS AM302) with a binary solvent system: the linear gradient of CH_3CN (0–100% CH_3CN for 40 min) in 0.1% aqueous TFA at a flow rate of 0.9 mL min^{-1} (temperature: 40 °C), detected at 230 nm.

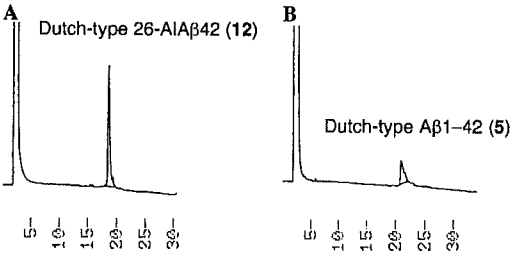


Figure 3. HPLC profiles of pure (A) Dutch-type 26-AIAβ42 (**12**) and (B) Dutch-type Aβ1–42 (**5**). The same quantity (2.3 nmol) of both peptides was applied to the HPLC column as a DMSO solution. Analytical HPLC was performed using a C18 reverse-phase column (4.6 mm × 150 mm; YMC Pack ODS AM302) with a binary solvent system: the linear gradient of CH_3CN (25–55% CH_3CN for 60 min) in 0.1% aqueous TFA at a flow rate of 0.9 mL min^{-1} (temperature: 40 °C), detected at 230 nm.

of **12**, no conversion to Dutch-type Aβ1–42 (**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 Aβ1–42 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-AIAβ42 (**8**–**14**) and Aβ1–42 (**1**–**7**)

26-AIAβ42	Aβ1–42	Water solubility		
		26-AIAβ42 (mg mL^{-1})	Aβ1–42 (mg mL^{-1})	ratio[b]
8[a]	**1**[a](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

[a] Data from Ref. 17.
[b] Ratio = solubility of 26-AIAβ42/solubility of Aβ1–42.

peak in preparative scale HPLC purification, it was laborious to separate the impurities and evaluate the purity as wild-type Aβ1–42 (**1**).[14,17]

The water solubility of mutant 26-AIAβ42s (TFA salt) was 15.0 mg mL^{-1} (**8**),[17] 4.4 mg mL^{-1} (**9**), 18.3 mg mL^{-1} (**10**), 5.1 mg mL^{-1} (**11**), 14.5 mg mL^{-1} (**12**), 16.2 mg mL^{-1} (**13**), and 8.1 mg mL^{-1} (**14**), higher than those in the corresponding mutant Aβ1–42s (**1**: 0.14,[17] **2**: 0.15, **3**: 0.95, **4**: 0.40, **5**: 0.93, **6**: 8.7, and **7**: 2.2 mg mL^{-1}) (Table 1). The water solubility ratios of isopeptides compared to the corresponding Aβ1–42s 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^{-1} (Table 1). This observation corresponds to our previous study regarding '*O*–*N* intramolecular acyl/acyloxy migration'-type water-soluble prodrugs of taxoids.[19i] 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 Aβ1–42 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_3CN, 60 min, Fig. 3A), while the corresponding Aβ1–42 **5** was eluted as a broad peak under the same elution conditions (Fig. 3B) as wild-type Aβ1–42 **1**.[14,17] These results indicate that the highly insoluble and agglutinative nature of Aβ1–42 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 Aβ1–42 **1** in our previous study.[17] Therefore, the *O*-acyl isopeptide method is a common strategy for increasing water-solubility. Recent reports by Carpino et al.[14l] and Mutter et al.[20] 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-AIAβ42 **12** was quantitatively converted to the corresponding Aβ1–42 **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_3CN for 40 min, 230 nm) of the newly appeared peak (Fig. 4) was identical to that of an independently synthesized Dutch-type Aβ1–42 **5** by a conventional method. Moreover, the

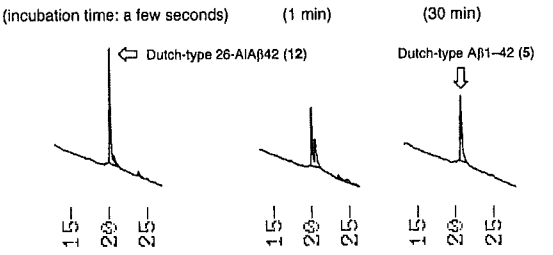


Figure 4. HPLC profiles of the conversion of Dutch-type 26-AIAβ42 (**12**) to corresponding Aβ1–42 (**5**) in PBS (pH 7.4) at 37 °C. Analytical HPLC was performed using a C18 reverse-phase column (4.6 mm × 150 mm; YMC Pack ODS AM302) with a binary solvent system: the linear gradient of CH_3CN (0–100% CH_3CN for 40 min) in 0.1% aqueous TFA at a flow rate of 0.9 mL min^{-1} (temperature: 40 °C), detected at 230 nm.

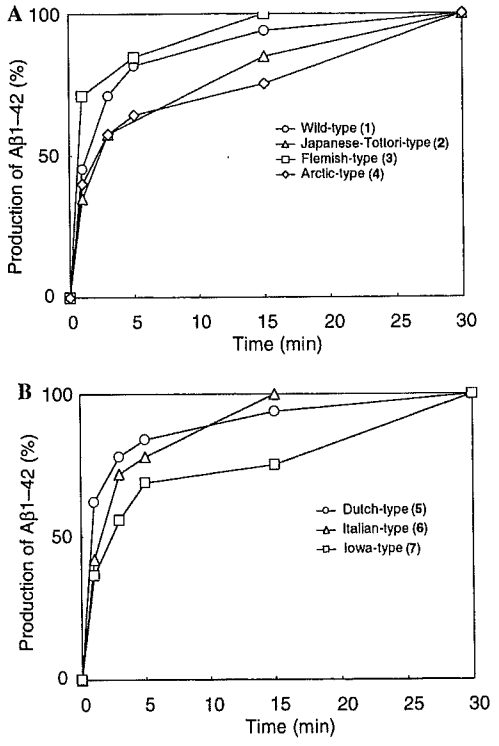


Figure 5. Time course of Aβ1–42 production in (A) wild-, Japanese-Tottori-, Flemish-, and Arctic-type isopeptides (B) Dutch-, Italian-, and Iowa-type isopeptides in PBS (pH 7.4) at 37 °C.

mass spectrometry analysis of this peak was identical to **5** (M_{calcd}: 4513.1; $M+H_{found}$: 4514.0). The rapid migration in **12** may be attributed to the lower steric hindrance of the Gly^{25} 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 Aβ1–42 mutant (for **2**; M_{calcd}: 4513.1; $M+H_{found}$ 4514.1; for **3**: M_{calcd}: 4500.0; $M+H_{found}$: 4501.4; for **4**: M_{calcd}: 4442.0; $M+H_{found}$: 4443.0; for **6**: M_{calcd}: 4513.1; $M+H_{found}$: 4513.9; for **7**: M_{calcd}: 4513.1; $M+H_{found}$: 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^{25}-Ser^{26}. These rapid migrations under physiological conditions would enable the production of intact monomer Aβ1–42 mutants 'in situ' to investigate the inherent biological functions of Aβ1–42 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 Aβ1–42 mutants. Namely, the isopeptides (1) suppressed the unfavorable nature of Aβ1–42 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 Aβ1–42 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 Aβ1–42. These results suggest that this method provides a new system in AD-related research, in which 26-*O*-acyl isoAβ1–42 (26-AIAβ42) can be stored in a solubilized form and rapidly produce intact Aβ1–42 in situ during biological experiments, which is useful for investigating the biological function of Aβ1–42 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 α-cyano-4-hydroxycinnamic acid as a matrix. Analytical HPLC was performed using a C18 reverse-phase column (4.6 mm × 150 mm; YMC Pack ODS AM302) with a binary solvent system: a linear gradient of CH_3CN in 0.1% aqueous TFA at a flow rate of 0.9 mL min^{-1} (temperature: 40 °C), detected at 230 nm. Preparative HPLC was carried out on a C18 reverse-phase column (20 mm × 250 mm; YMC Pack ODS SH343-5) with a binary solvent system: a linear gradient of CH_3CN in 0.1% aqueous TFA at a flow rate of 5.0 mL min^{-1} (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^{α}-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×) and the completeness of each coupling was verified by the Keiser test. N^{α}-Fmoc deprotection was carried out by treatment with piperidine (20% v/v in DMF) (2 mL, 1 min × 1 and 20 min × 1), followed by washing with DMF (1.5 mL, 10×) and chloroform (1.5 mL, 5×). 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×) 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×). The resultant peptides were dissolved or suspended in water and lyophilized for at least 12 h. The peptides were reduced using NH_4I–dimethylsulfide in TFA/H_2O (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_3CN 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 β peptide (Aβ)1–42 (E22Q, 5) by '*O*-acyl isopeptide method'

(1) 26-*O*-Acyl isoAβ1–42(E22Q, **12**)

After protected Aβ27–42-resin (chlorotrityl 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 μL, 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 μL, 1.4 mmol)–DMAP (3.5 mg, 0.03 mmol) method in CH_2Cl_2 (1.5 mL) for 4 h[18b] (2×). 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 μL)–thioanisole (261 μL)–H_2O (261 μL) 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_2O (0.5 mL) in the presence of NH_4I (6 mg, 0.04 mmol) and dimethylsulfide (3 μL, 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 μm filter unit, applied to preparative HPLC, and eluted using a 0.1% aqueous TFA–CH_3CN. 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_{calcd}: 4513.1; $M+H_{found}$: 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_{calcd}: 4513.1; $M+H_{found}$: 4514.0; HPLC analysis at 230 nm: purity was >95%. The retention time on HPLC (0–100% CH_3CN for 40 min, 230 nm) of **5** was identical to that of Dutch-type Aβ1–42 synthesized independently by the conventional method.

5.4. Aβ1–42 (D7N, 2), (A21G, 3), (E22G, 4), (E22K, 6), (D23N, 7) by '*O*-acyl isopeptide method'

(1) 26-*O*-Acyl isoAβ1–42 (D7N, **9**), (A21G, **10**), (E22G, **11**), (E22K, **13**), (D23N, **14**)

26-*O*-Acyl isoAβ1–42 (**9–11**, **13**, and **14**) were synthesized in a similar manner to **12**. Chemical data for **9**: yield: 26.2%; MALDI-MS (TOF): M_{calcd}: 4513.1; $M+H_{found}$: 4514.5; HPLC analysis at 230 nm: purity was higher than 95%; for **10**: yield: 26.4%; MALDI-MS (TOF): M_{calcd}: 4500.0; $M+H_{found}$: 4501.3; HPLC analysis at 230 nm: purity was higher than 95%; for **11**: yield: 34.0%; MALDI-MS (TOF): M_{calcd}: 4442.0; $M+H_{found}$: 4442.9; HPLC analysis at 230 nm: purity was higher than 95%; for **13**: yield: 24.6%; MALDI-MS (TOF): M_{calcd}: 4513.1; $M+H_{found}$: 4514.6; HPLC analysis at 230 nm: purity was higher than 95%; for **14**: yield: 21.3%; MALDI-MS (TOF): M_{calcd}: 4513.1; $M+H_{found}$: 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_{calcd}: 4513.1; $M+H_{found}$: 4514.1; for **3**: M_{calcd}: 4500.0; $M+H_{found}$: 4501.37; for **4**: M_{calcd}: 4441.98; $M+H_{found}$: 4443.0; for **6**: M_{calcd}: 4513.1; $M+H_{found}$: 4513.9; for **7**: M_{calcd}: 4513.1; $M+H_{found}$: 4514.2; HPLC analysis of **2–4**, **6**, and **7** at 230 nm: purity was >95%. The retention time on HPLC (0–100% CH_3CN for 40 min, 230 nm) of formed **2–4**, **6**, and **7** was identical to that of each independently synthesized Aβ1–42 mutant by the conventional manner.

5.5. Amyloid β peptide (Aβ)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*-diisopropylethylamine (DIPEA, 26.2 μL, 0.15 mmol) in 1,2-dichloroethane (1.5 mL). After washing with DMF (1.5 mL, 5×), capping was performed with MeOH (200 μL) in the presence of DIPEA (52.5 μL, 0.3 mmol) in DMF for 20 min. After washing with DMF (1.5 mL, 5×), DMF–H_2O (1:1, 1.5 mL, 5×), $CHCl_3$ (1.5 mL, 2×), and MeOH (1.5 mL, 2×) 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 μL, 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 μL)–thioanisole (181 μL)–H_2O (181 μL) 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_2O (0.5 mL) in the presence of NH_4I (6 mg, 0.04 mmol), and dimethylsulfide (3 μL, 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 μm filter unit, applied to preparative HPLC, and eluted using 0.1% aqueous TFA–CH_3CN. 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_{calcd}: 4513.0; $M+H_{found}$: 4514.2; HPLC analysis at 230 nm: purity was >94%.

5.6. Aβ1–42 (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_{calcd}: 4513.1; $M+H_{found}$: 4514.0; HPLC analysis at 230 nm: purity was higher than 94%; for **3**: yield: 0.5%; MALDI-MS (TOF): M_{calcd}: 4500.0; $M+H_{found}$: 4501.4; HPLC analysis at 230 nm: purity was higher than 95%; for **4**: yield: 10.1%; MALDI-MS (TOF): M_{calcd}: 4442.0; $M+H_{found}$: 4442.9; HPLC analysis at 230 nm: purity was higher than 95%; for **6**: yield: 22.7%; MALDI-MS (TOF): M_{calcd}: 4513.1; $M+H_{found}$: 4514.2; HPLC analysis at 230 nm: purity was higher than 95%; for **7**: yield: 6.7%; MALDI-MS (TOF): M_{calcd}: 4513.1; $M+H_{found}$: 4513.9; HPLC analysis at 230 nm: purity was higher than 95%. The elution profiles of Aβ1–42 mutants in a slow gradient system (25–55% CH_3CN for 60 min) were ill-defined; however, TOF-MS indicated that only a single species was present. Glabe and coworkers[14a] and Johnson and coworkers[14b] have commented upon the chromatographic behavior of Aβ, 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 μm filter unit,