Further applications of the classical amide coupling reagents: microwave assisted esterification on solid-phase

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CONTENTS

EXPERIMENTAL

1. General method

Commercially available reagents and solvents were used without further purification. COMU ((1-Cyano-2-ethoxy-2-oxoethylidenaminooxy) dimethylamino-morpholino-carbenium hexafluorophosphate) was a gift from Luxembourg Bio Technologies (Israel). The Wang linker PS (polystyrene) resin, *N*α-[9-(Fluorenylmethoxy)carbonyl (Fmoc)]-L-amino acids, 1-(Mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole (MSNT) and Oxyma pure (Ethyl cyano(hydroxyimino)acetate) were obtained from Novabiochem (Laufelfingen, Swizerland). HBTU (N-[(1H-benzotriazol-1-yl)-(dimethylamino)methylene]-N-methylmethanaminium hexafluorophosphate N-oxide), DMF (*N*,*N*-dimethylformamide) and CH_2Cl_2 were purchased from Watanabe Chemical Industries, Ltd. (Hiroshima, Japan).

Hydroxybenzotriazole monohydrate (HOBt) and *N*,*N*-diisopropylethylamine (DIEA)

were purchased from Kokusan chemical Co.,Ltd. (Yokohama, Japan). Oxyma (Ethyl (hydroxyimino)cyanoacetate), *N,N'*-Diisopropyl carbodiimide (DIC), 2,2,2-trifluoroacetic acid (TFA), CH_3CN and NMI (N-Methylimidazole) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). DMAP (4-dimethylaminopyridine) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). All solid-phase reactions were performed manually in a polypropylene tube equipped with a filter (LibraTube, Hipep Laboratories, Kyoto, Japan). Microwave-assisted coupling and deprotection reactions on resins were carried out on an EYELA microwave synthesizer Wave Magic (MWS-1000A, Tokyo Rikakikai Co., LTD., Tokyo, Japan), in which the reaction temperature was kept at 50ºC with a wattage range of 0-100 W.

Analytical Reverse Phase-Ultra Performance Liquid Chromatography (RP-UPLC) was conducted by using a Waters Acquity Ultra Performance LC system equipped with binary solvent delivery pump, an auto sampler and a UV detector [BEN®C_{18} column (1.7 mm, 2.1 × 50 mm, Waters), flow rate of 0.2 mL/min]. The conditions are described for each compound. A gradient of water with 0.1% TFA (eluent A) and acetonitrile with 0.1% TFA (eluent B) were used for the mobile phase. The conditions are described for each compound. Mass spectra were obtained on a Bruker Daltonics Ultraflex MALDI-TOF/MS instrument. The matrix used is a solution of DHB in a 1:1 mixture of H_2O–CH_3CN in 0.1% TFA.

2. Peptide synthesis

Manual solid-phase peptide elongation and other solid-phase manipulations were carried out in polypropylene syringes, each fitted with a porous disk. Solvent and soluble reagents were removed by suction. Previous washing of Wang linker resin polystirene were performed with DMF (5×1 min) and CH_2Cl_2 (5×1 min). Washings between deprotection, coupling and subsequent deprotection step were carried out at room temperature (r.t.) with DMF (3 × 1 min), CH_2Cl_2 (3 × 1 min) and DMF (3 × 1 min). *N*-Acetylation was carried out with Ac_2O—DIEA—DMF (10:5:85) at r.t. for 3 min. Fmoc removal was carried out with pip—DMF (1:5) (1 × 3 min, MW assisted). The peptide chains were assembled by the conventional method: the sequential coupling of amino acids (4 equiv.) with HBTU—HOBt—DIEA (4:4:6) in DMF for 10 min assisted by MW energy at 50 °C.

3. Fmoc loading test

Deprotection with piperidine gives the fulvene – piperidine adduct which can be determined by quantitative spectrophotometric at 290 nm (E_{280} = 5800). Loading calculation was determined by the Abs at 290nm of the collecting washings after deprotection in DMF. Each determination was done in triplicate.

4. Esterification methods

MSNT

Washings and deprotection were performed as described above. The coupling of first amino acids (4 equiv.) was carried with MSNT－NMI (4:8) in CH_2Cl_2 for 30 min at room temperature. *N*-Acetylation and assembly of peptide chain were carried out by the conventional method described above. The coupling rate was analyzed by the Fmoc loading test.

DIC/DMAP

Washings and deprotection were performed as described above. The coupling of amino acids (4 equiv.) was carried with DIC (3.5 equiv.) and DMAP (cat.) in CH_2Cl_2 for overnight coupling reaction at room temperature. *N*-Acetylation and assembly of peptide chain were carried out by the conventional method described above. The coupling was analyzed by Fmoc loading test.

HBTU/HOBt

The coupling of first amino acid (4 equiv.) was carried out with HBTU－HOBt－DIEA (6:6:8) in DMF for indicated time assisted by MW energy. The coupling rate was analyzed by the Fmoc loading test.

5. Racemization Assay

The peptides (Fmoc-Gly-Phe-His-OH) were cleaved from the resin by treatment with TFA－H_2O (9:1) for 1 h at room temperature. A ratio of 100 μ L of cleavage cocktail per mg of resin was performed. After cleavage reaction, peptides were precipitated by adding cold *tert*-butylmethyl ether. Following centrifugation, the solution decanted and this process was repeated three times. Finally, peptides were dissolved in H_2O－CH_3CN (1:1) and lyophilized. The peptides were dissolved in H_2O－CH_3CN (7:3) and analyzed by analytical RP-UPLC [isocratic of Eluent A:Eluent B (7:3) for 15 min; UV detection was performed at 280nm] and by MALDI-TOF/MS, m/z calculated for FmocGFH-OH and FmocGFh-OH is 581.2274. Result of MSNT esterification method are:

Fmoc-GFH-OH, t_R = 12.927 min, 89.26%; m/z found, 582.1885 $[M+H]^+$ and Fmoc-GFh-OH, t_R = 11.672 min, 10.74%; m/z found 582.2538 $[M+H]^+$ (Figure S1). Characterization of compounds after DIC/DMAP method are: Fmoc-GFH-OH, t_R = 12.913 min, 71.38%; m/z found, 582.1891 $[M+H]^+$ and Fmoc-GFh-OH, t_R = 11.565 min, 28.62%; m/z found 582.2601 $[M+H]^+$ (Figure S2). Characterization of tripeptide by HBTU/HOBt method: Fmoc-GFH-OH, t_R = 12.748 min, 84.01%; m/z found, 582.2870 $[M+H]^+$ and Fmoc-GFh-OH, t_R = 11.447 min, 15.99%; m/z found 582.2206 $[M+H]^+$ (Figure S3). And finally, by COMU/Oxyma method: Fmoc-GFH-OH, t_R = 12.512 min, 73.55%; m/z found, 582.2153 $[M+H]^+$ and Fmoc-GFh-OH, t_R = 11.193 min, 26.45%; m/z found 582.2121 $[M+H]^+$ (Figure S4).

6. Depsipeptide Synthesis

Manual solid-phase peptide elongation and other solid-phase manipulations were carried out in polypropylene syringes, each fitted with a porous disk. Solvent and soluble reagents were removed by suction. Previous washing of the Tentagel S RAM resin were performed with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min). Washings between deprotection, coupling and subsequent deprotection step were carried out at room temperature with DMF (3 × 1 min), CH_2Cl_2 (3 × 1 min) and DMF (3 × 1 min). Fmoc removal was carried out with pip－DMF (1:5) (1 × 3 min, MW assisted). The Boc-Ser(Trt)-Val-Val-NH-resin was synthesized by the conventional method: the coupling of amino acids (4 equiv.) with HBTU－HOBt－DIEA (4:4:6) in DMF for 10 min assisted by MW energy. *N*-Acetylation was carried out with Ac_2O－DIEA－DMF (10:5:85) at r.t. for 3 min. After incorporation of Boc-Ser(Trt)-OH, Trt group removal was carried out with TFA－CH_2Cl_2－TIS (1:94:5) (3×2 min) at room temperature. Completeness of reaction was checked by treating a small sample of resin with TFA－CH_2Cl_2 (1:1) and repeat Trt removal reaction until a colorless solution was obtained (as Trt deprotection provides a red color to the solution).[1] Washing was carried out with CH_2Cl_2 (5×1 min). Subsequent coupling with Fmoc-Val-OH to the β-hydroxy group of Ser was performed in a similar manner to that described in HBTU/HOBt esterification method, followed by deprotection of Fmoc and the coupling of another Val residue with conventional amide formation method. After Fmoc renoval and final acetylation step, peptide was cleaved from the resin by treatment with TFA－thioanisole－m-cresol－H_2O (92.5:2.5:2.5:2.5) for 1 h at room temperature. A ratio of 100 μL of cleavage cocktail per mg of resin was added. After cleavage reaction, depsipeptide was precipitated by adding cold *tert*-butylmethyl ether. Following centrifugation, the solution decanted and this process was repeated three times. Finally, depsipeptide was

dissolved in H_2O-CH_3CN (1:1) and lyophilized. The cleaved peptide was dissolved in H_2O-CH_3CN (8:2) and analyzed by analytical RP-UPLC[gradient: eluent A−eluent B (8:2) to eluent A−eluent B (4:6) for 10 min; UV detection was performed at 220nm]. Major peak was collected and mass was confirmed by MALDI-TOF MS, m/z calculated for depsi Ac-V*VS*VV-OH is 542.3428. depsi Ac-V*VS*VV-OH, t_R = 6.047 min, 33.87%; found, 565.3976 $[M + Na]^+$, 581.4424 $[M + K]^+$ (Figure S5).

7. Figures of SI

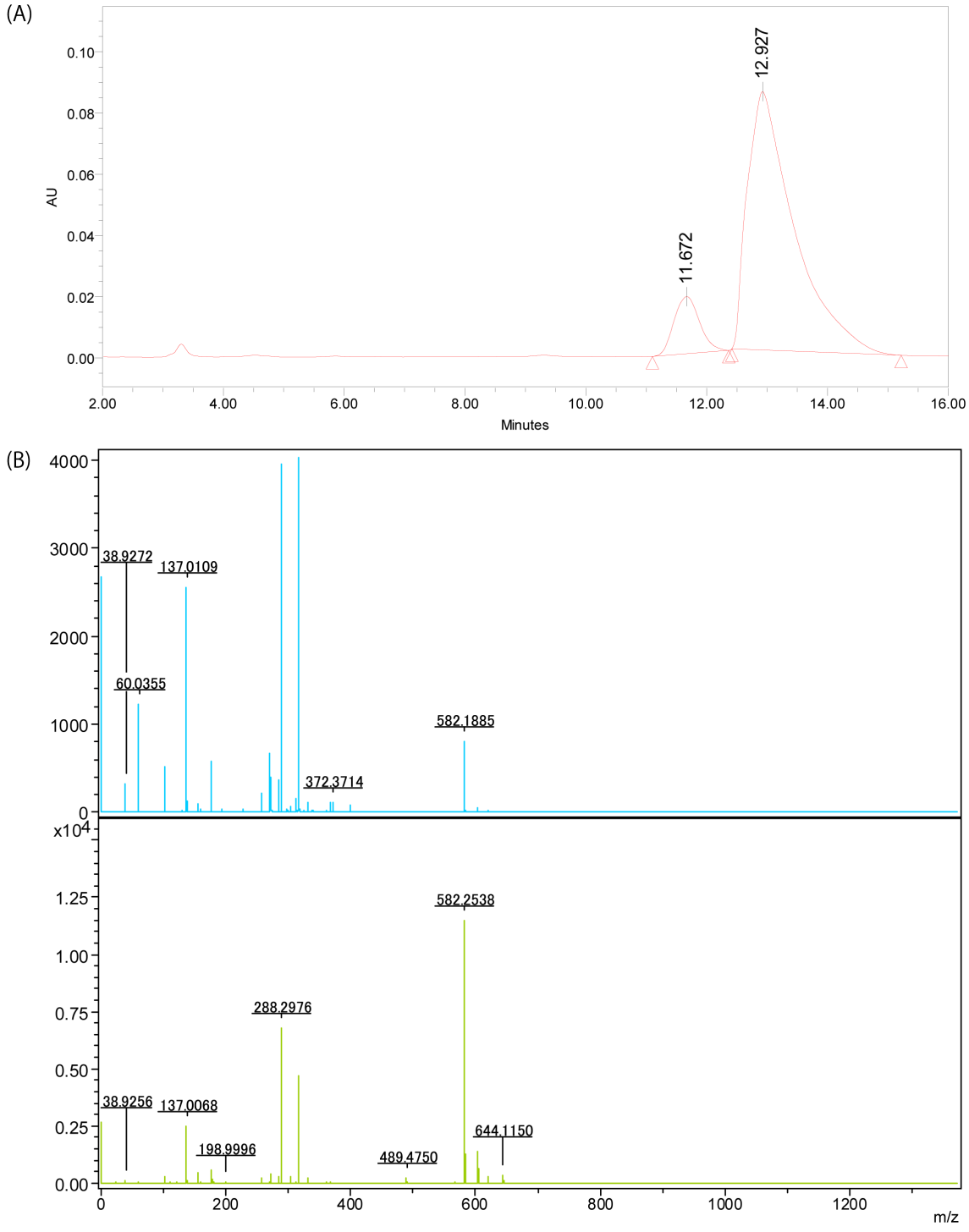

Figure S1. Characterization of Fmoc-Gly-Phe-His-OH. Esterification by MSNT method.
(A) RP-UPLC chromatogram (Isocratic of 30% Eluent B in Eluent A for 15 min) at 280nm
(B) MALDI-TOF MS spectra of isolated compounds Fmoc-GFh-OH and Fmoc-GFH-OH (m/z calcd. 581.2274; found 582.1885 $[M+H]^+$ and 582.2538 $[M+H]^+$)

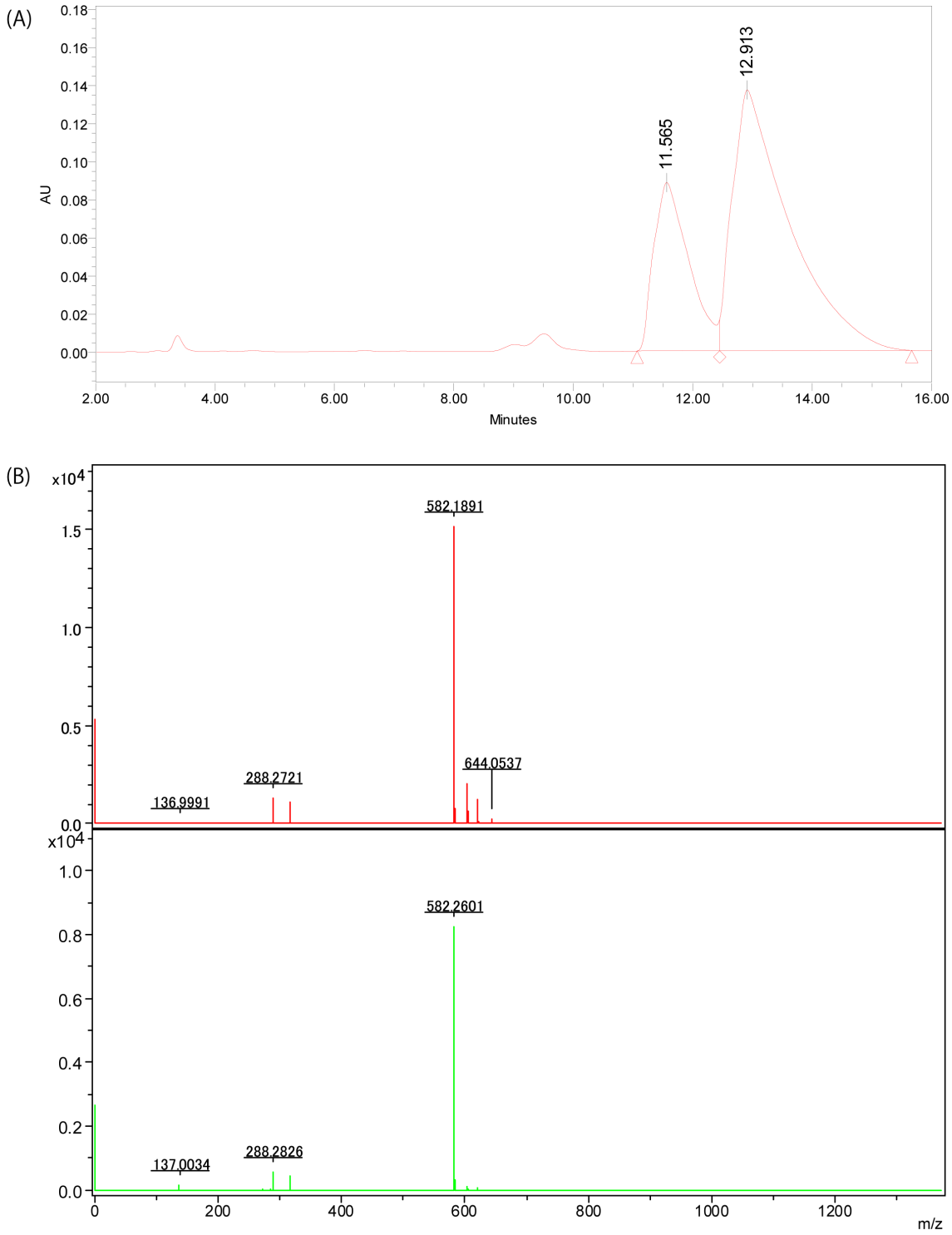

Figure S2. Characterization of Fmoc-Gly-Phe-His-OH. Esterification by DIC method.

(A) RP-UPLC chromatogram (Isocratic from 30% of Eluent B in Eluent A for 15 min) at 280nm.

(B) MALDI-TOF MS spectra of isolated compounds Fmoc-GFh-OH and Fmoc-GFH-OH (m/z calcd. 581.2274; found 582.1891 $[M+H]^+$ and 582.2601 $[M+H]^+$)

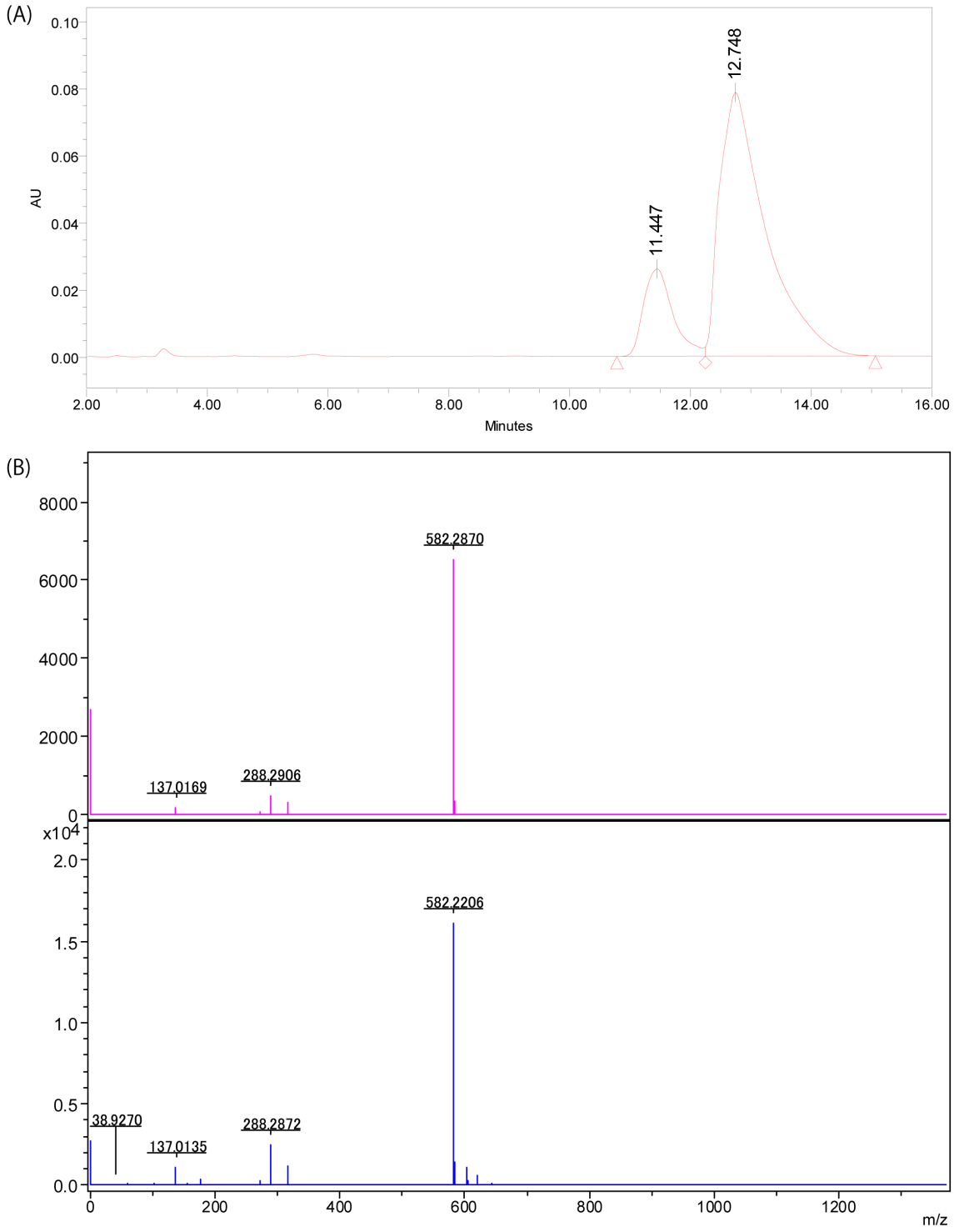

Figure S3. Characterization of Fmoc-Gly-Phe-His-OH. Esterification by HBTU method.

(A) RP-UPLC chromatogram (Isocratic from 30% of Eluent B in Eluent A for 15 min) at 280nm

(B) MALDI-TOF MS spectra of isolated compounds Fmoc-GFh-OH and Fmoc-GFH-OH (m/z calcd. 581.2274; found 582.2870 $[M+H]^+$ and 582.2206 $[M+H]^+$)

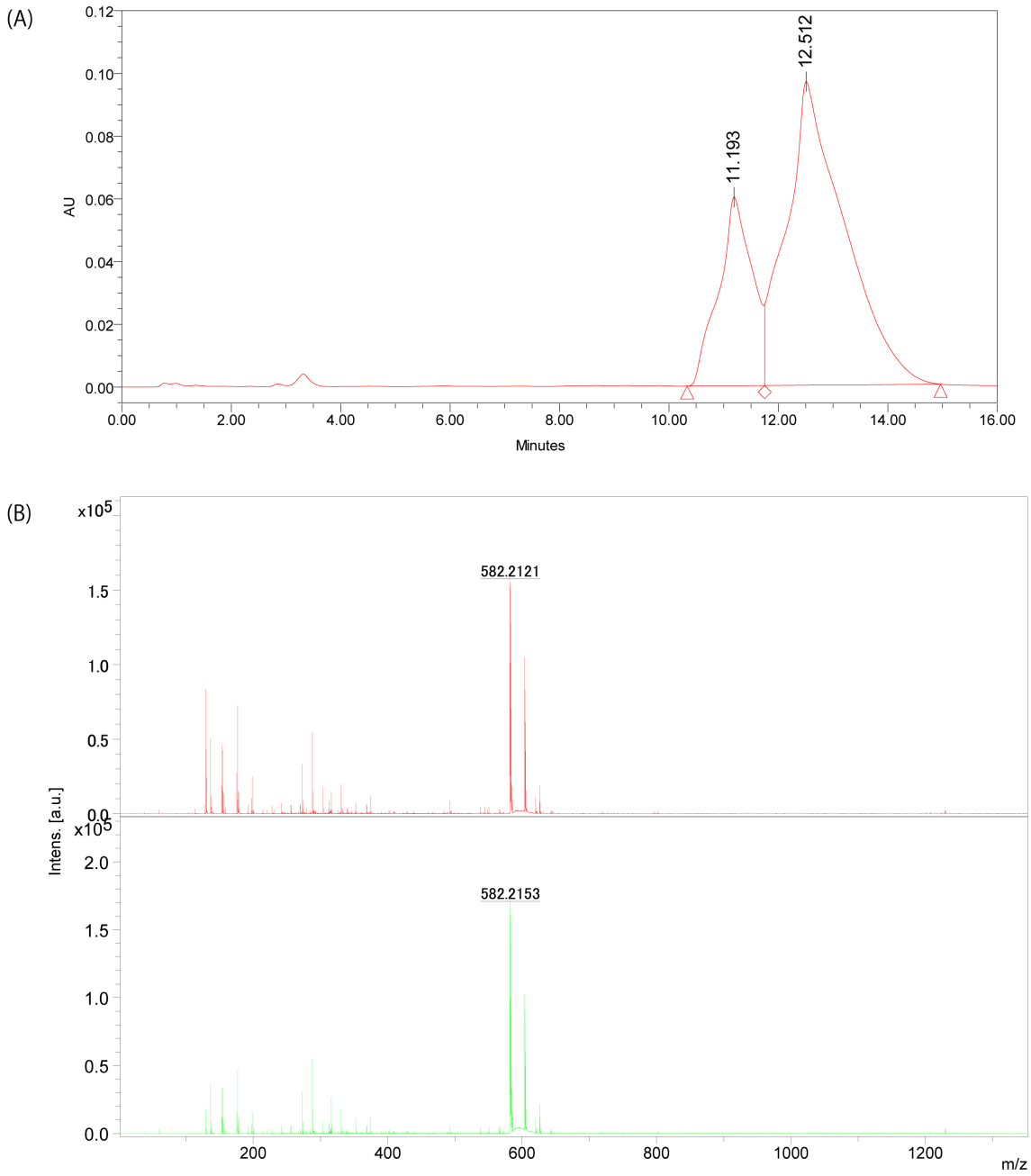

Figure S4. Characterization of Fmoc-Gly-Phe-His-OH. Esterification by COMU method.

(A) RP-UPLC chromatogram (Isocratic from 30% of Eluent B in Eluent A for 15 min) at 280nm

(B) MALDI-TOF MS spectra of isolated compounds Fmoc-GFh-OH and Fmoc-GFH-OH (m/z calcd. 581.2274; found 582.2121 $[M+H]^+$ and 582.2153 $[M+H]^+$)

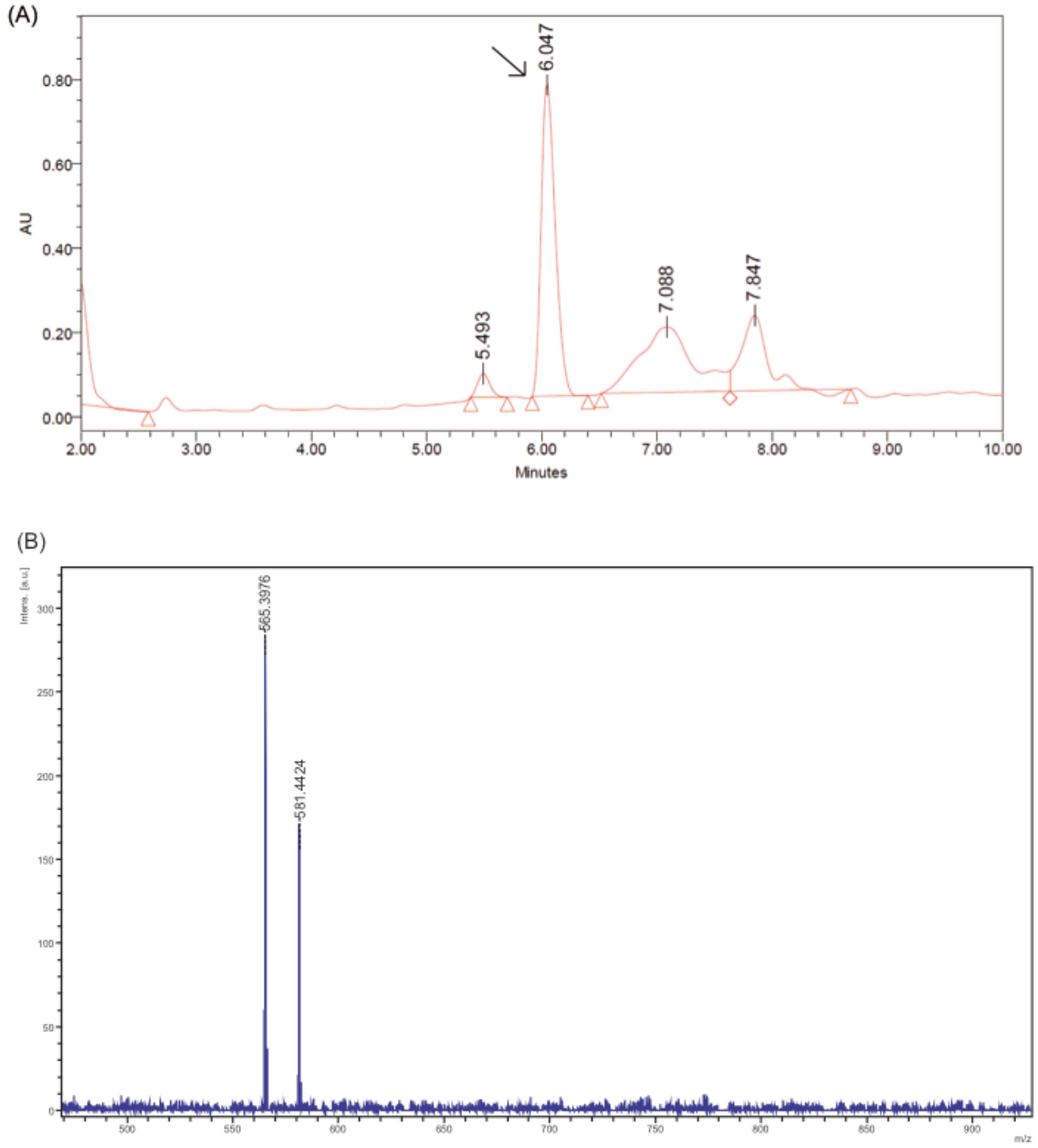

Figure S5. Characterization of the depsipeptide Ac-Val-*Val-Ser*-Val-Val-NH_2 (**1**). Synthesis based on HBTU/HOBt under microwave irradiation.

(A) RP-UPLC chromatogram (Gradient Eluent B:Eluent A = (2:8) to Eluent B:Eluent A = (6:4) for 10 min) at 220nm

(B) MALDI-TOF MS spectrum of isolated compound Depsi-Ac-V*VS*VV-NH_2 (m/z calcd. 542.3428; found 565.3976 $[M+Na]^+$ and 581.4424 $[M+K]^+$)

8. References

1) Carpino L. A. *et al. Tetrahedron Lett*, 2004; **45**: 7519–7523