RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

Journal Name

RSCPublishing

COMMUNICATION

Solid Phase Synthesis of α-Amino Squaric Acid-Containing Peptides

Cite this: DOI: 10.1039/xoxxoooooox
Received ooth January 2012,
Accepted ooth January 2012
DOI: 10.1039/xoxxoooooox
www.rsc.org/

Kentaro Maeda,[a] Yuuichi Kiniwa,[a] Yasufumi Ohfune,[a] Shinichi Ishiguro,[b] Koichi Suzuki,[b] Kazuya Murata,[c] Hideaki Matsuda,[c] and Tetsuro Shinada*[,a]

A new method has been developed for the synthesis of 3-(1-aminoalkyl)-4-hydroxycyclobut-3-ene-1,2-dione [α-amino squaric acid (α-Asq)]-containing peptides using solid phase peptide synthesis according to an Fmoc protecting group strategy. FmocHN-Gly-[α-Asq]-O*i*-Pr was successfully used as a coupling unit in this method, which allowed for the construction of α-Asq-containing hexapeptide libraries including Sq-Gly and Sq-Phe units using the Wang-resin. Peptides containing the α-Asq moiety exhibited inhibitory activity towards a digestive enzyme.

The solid phase synthesis of peptide using the Fmoc protecting group (i.e., the Fmoc strategy) represents a robust technology for the rapid construction of various peptides, which is also amenable to the automated synthesis of peptide libraries.[1] The application of this technique to the synthesis of peptides containing amino acid isosteres has recently attracted considerable levels of attention because the resulting peptide analogues exhibit improved levels of potency and selectivity towards biological targets, as well as enhanced metabolic and chemical stabilities compared with the corresponding unmodified peptides. Several amino acid isosteres have been developed for use in the solid phase synthesis of peptide analogues.[2]

We recently reported 3-(1-aminoalkyl)-4-hydroxycyclobut-3-ene-1,2-dione [α-amino squaric acid (α-Asq)] (**1**) as a novel amino acid analogue, where the carboxylic acid moiety of the α-amino acid had been replaced with a 4-hydroxy-2,3-dioxocyclobut-1-enyl (Sq) group (Figure 1).[3] The Sq group and squaric acid **2** have been studied extensively as carboxylic acid surrogates in medicinal chemistry, bioorganic chemistry, chemical biology, and materials chemistry.[3] It was envisaged that the incorporation of α-Asq into peptides would allow for the preparation of novel α-Asq-containing peptides, because the inherent reactivity of the Sq group would allow this moiety to react with various amines to form amide like N-Sq bonds.[3,4] In our previous study, we reported for the first time the synthesis of the Sq-Gly-containing Leu-enkephalin analogue **3** (Figure 1),[4b] which exhibited promising binding activities towards opioid receptors. This compound was also found to be stable under acidic conditions (i.e., 30% HBr/AcOH), as well as being stable to the conditions required for its purification by HPLC (i.e., H_2O/MeCN/TFA), and its subsequent analysis by NMR spectroscopy in DMSO-d_6 and D_2O.[4b] These observations led us to explore the possibility of developing a method for the rapid construction of α-Asq-containing peptides using the Fmoc strategy. Herein, we report the first solid phase synthesis of α-Asq-containing peptides and their application to the construction of peptide analogue libraries containing the α-Asq unit.

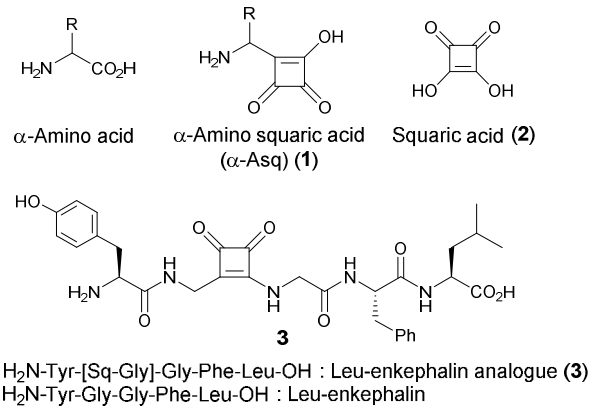
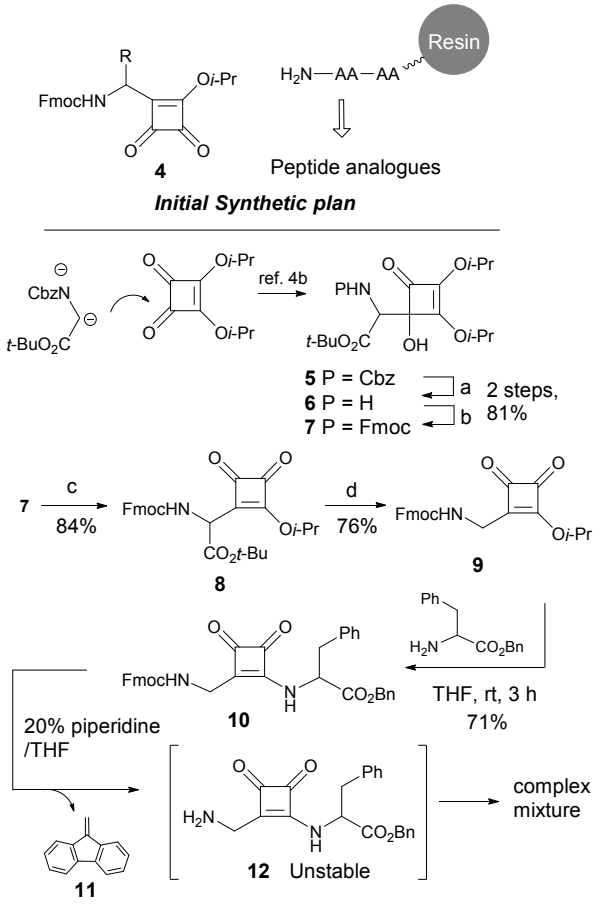


Figure 1. Structures of α-amino acid, α-Asq (**1**), squaric acid (**2**), and α-Asq-containing Leu-Enkephalin (**3**).

FmocHN-[α-Asq]-O*i*-Pr **4** was selected as a coupling unit for the Fmoc strategy used in this study (Scheme 1). The synthesis of FmocHN-[Sq-Gly]-O*i*-Pr **9** was commenced with *N*-Cbz-hydroxycyclobutenone **5**.[4b] Removal of the Cbz group from **5** followed by protection of the resulting free amino group with the Fmoc group gave **7** in 81% yield, which was converted to *N*-Fmoc α-Sq glycine **8** in 84% yield using the conventional condition in the presence of a small amount of 12*N* HCl.[3,4] Subsequent removal of the *t*-butyl group in compound **8** provided the corresponding carboxylate, which underwent a spontaneous Sq group-promoted decarboxylation reaction[4] *in situ* to give FmocHN-[Sq-Gly]-O*i*-Pr **9** in 76% yield.

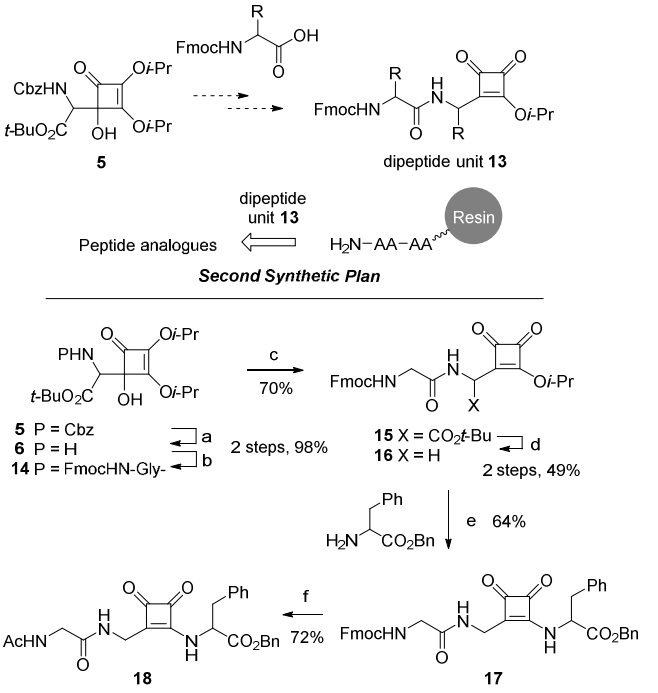
The Fmoc strategy used in the current study consists of a series of sequential transformations, including (i) linkage of the Fmoc-protected amino acids (FmocHN-AA-OH) to the acid-labile linker on solid phase; (ii) removal of the Fmoc protecting group from the AA; (iii) condensation of FmocHN-AA-OH; (iv) repetition of steps (ii) and (iii) to elongate the peptide chain; and (v) treatment of the resulting peptide with acid to cleave the peptide from the resin. We initially investigated the compatibility of Fmoc-[Sq-Gly]-O*i*-Pr **9** under the

proposed reaction conditions prior to the solid phase synthesis using a model system involving the dipeptide analogue FmocHN-[Sq-Gly]-Phe-OBn **10**. Treatment of **9** with H_2N-Phe-OBn gave **10** in 71 % yield (Scheme 2). Our initial attempt to remove the Fmoc group in **10** using 20% piperidine in THF resulted in a complex mixture. We also investigated the use of a variety of different reagents and conditions [e.g., Et_2NH, DBU, 2,2,6,6-tetramethyl piperidine, lower temperature, different concentrations, *in situ* trapping with Boc_2O and Ac_2O], but none of these reactions resulted in the formation of the desired amine **12**. 9-Methylidenefluorene (**11**) was detected as major by-product in the complex mixture resulting from this reaction, which indicated that the Fmoc group was being liberated during the course of the reaction to give amine **12**, which must have underwent rapid decomposition.



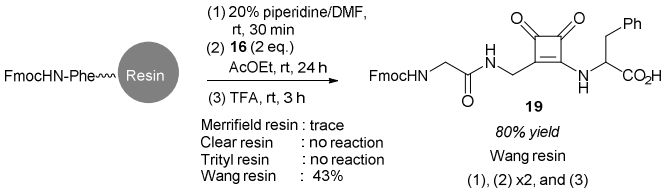
Scheme 1. Reaction Conditions: (a) H_2/Pd-C (20 wt%), MeOH, rt, 3 h; (b) FmocOSu (1 eq.), MeCN, rt, 4 h; (c) 12*N* HCl (1 eq.), CH_2Cl_2, rt, 3 h; (d) TFA (30 eq.), CH_2Cl_2, 0 °C to rt, 12 h.

It was envisaged that the protection of the free amine group as an amide such as dipeptide unit **13** would overcome the decomposition issues observed above and allow for the use for the solid phase synthesis (Scheme 2). FmocHN-Gly-[Sq-Gly]-O*i*-Pr **16** was synthesized from compound **5** according to the route shown below.[4a] Compound **6** was coupled with FmocHN-Gly-OH using EDCI to give **14** in 98% yield. Compound **14** was then converted to **16** in a similar manner to that used for the synthesis of **9** via a Sq group-promoted decarboxylation reaction. FmocHN-Gly-[Sq-Gly]-O*i*-Pr **16** was then condensed with H_2N-Phe-OBn to give the model tripeptide analogue **17** in 64% yield. Compound **17** was treated with Et_2NH to allow for the removal of the Fmoc protecting group, and the resulting amine was acetylated with Ac_2O to give the corresponding amide **18** in 72% yield, without decomposition of the free amine (Scheme 2).



Scheme 2. Reaction Conditions: (a) H_2/Pd-C (20 wt%), MeOH, rt, 3 h; (b) FmocHN-Gly-OH (1 eq.), EDCI (1 eq.), THF, rt, 3 h; (c) 12*N* HCl (1 eq.), CH_2Cl_2, rt, 3 h; (d) TFA (30 eq.), CH_2Cl_2, 0 °C to rt, 12 h; (e) H_2N-Phe-OH (3 eq.), THF, rt, 3 h; (f) 20% Et_2NH/THF, rt, 0.5 h then Ac_2O, THF, rt, 0.5 h.

With a suitably stable linkage unit in hand, we proceeded to evaluate the use of solid-supported **16** in a series of sequential transformations, including (i) removal of the Fmoc group of the FmocHN-Phe-functionalised Merrifield resin; (ii) treatment of the resulting amine with 2 equivalents of **16**; and (iii) cleavage of the resulting peptide from the resin by treatment with TFA (Scheme 3).

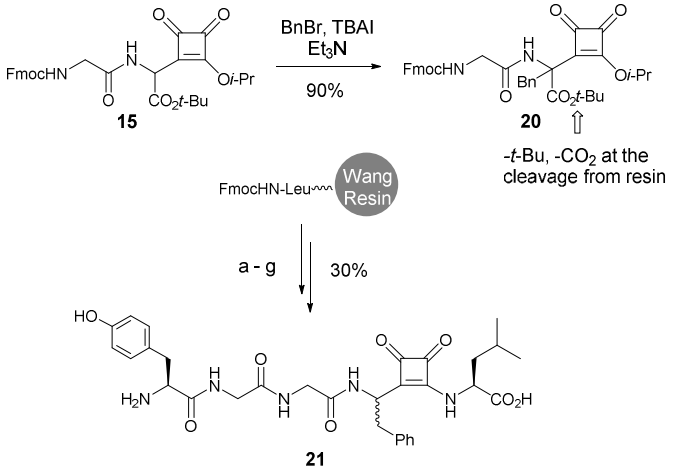


Scheme 3. Solid Phase Support of Dipeptide Unit **16**.

The yield of FmocHN-Gly-[Sq-Gly]-Phe-OH **19** following the cleavage of the product from the resin was used to evaluate the linkage efficiency of the peptide to the resin. Unfortunately, however, this protocol only resulted in the formation of a trace amount of **19**. Increasing the amount of **16**, as well as extending the reaction period or switching to trityl resin and Clear resin® resulted in similarly low yields of the product. Following an extensive period of investigation,

we found that the Wang resin® gave the best results of all of the resins tested, with **19** being isolated in 43% yield following a single loading of **16**. The coupling efficiency could be further improved by twice repeating the loading of **16**. Under the optimized conditions, **19** was isolated in 80% yield following its cleavage from the resin.

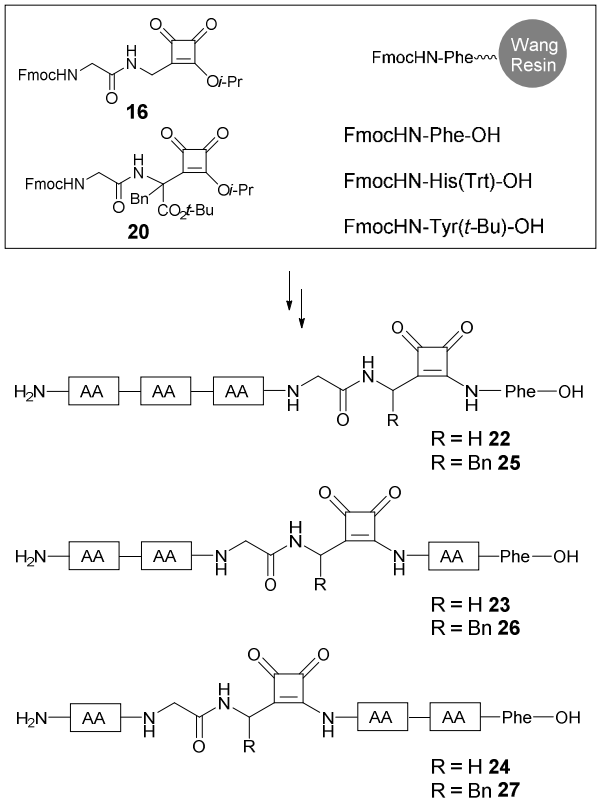
The α-substituted α-Asq, Sq-Phe, could also be incorporated into the peptide using the Fmoc strategy. To shorten the number of synthetic steps required of this protocol, we investigated the use of the dipeptide unit **20** bearing a -COO*t*-Bu group at the α-position of Sq-Phe as the coupling unit.[5] It was envisaged that the -COO*t*-Bu group could be readily removed under the conditions used for the cleavage of the peptide from the resin using TFA, which would also allow for the removal of the *t*-Bu group and decarboxylation.



Scheme 4. Synthesis of Sq-Phe-containing Peptide. Reaction Conditions: (a) 20% piperidine/DMF, rt, 15 min; (b) **20** (4 eq.), DIEA (2 eq.), AcOEt, rt, 60 h; (c) 20% Et_2NH/DMF, rt, 15 min; (d) FmocHN-Gly-OH (4 eq.), HOBt (4 eq.), HBTU (3.6 eq.), DIEA (8 eq.), DMF, rt, 1 h; (e) 20% Et_2NH/DMF, rt, 15 min; (f) FmocHN-Tyr(O*t*-Bu)-OH (4 eq.), HOBt (4 eq.), HBTU (3.6 eq.), DIEA (8 eq.), DMF, rt, 1 h; (g) TFA, rt, 3 h.

Compound **20** was prepared from **15** in 90% yield following a benzylation reaction[4c] in the presence of Et_3N. The suitability of **20** for use in the solid phase synthesis protocol was evaluated by the synthesis of Leu-enkephalin analogue **21**. Treatment of the Sq-Phe-containing dipeptide unit **20** (4 equiv) with the Wang resin in the presence of DIEA (2 equiv) allowed for the dipeptide to be efficiently immobilized on the resin. In this case, excess amount of **20** could be recovered cleanly from the filtrate after coupling reaction. The remaining two amino acid residues Gly and Tyr were successfully condensed with the resin-supported peptide using HOBt (4 equiv) and HBTU (3.6 equiv). Treatment of the peptide analogue-linked resin with TFA provided the Sp-Phe-containing enkephalin analogue **21** in an overall yield of 30% (Scheme 4).

The synthetic strategy described above was extended to the construction of an α-Asq-containing peptide library using the dipeptide units **16** and **20** (Schemes 5). Starting from FmocHN-Phe-Wang resin, a mixture of FmocHN-Phe-OH, FmocHN-Tyr(O*t*-Bu)-OH, FmocHN-His(Trt)-OH and dipeptide unit **16** were linked on the solid phase. The three peptide libraries **22**, **23**, and **24**, which differed only in the positioning of the Gly-[Sq-Gly] unit, were generated in a parallel manner. AA in the each library represents either Phe, Tyr, or His. The composition of each peptide library (including 27 hexapeptide analogues) was confirmed by MALDI-TOF MS analysis (Scheme SI-1). The Sq-Phe-containing peptide libraries **25**, **26**, and **27** were successfully synthesized in a similar manner (Scheme SI-2).



Scheme 5. Syntheis of α-Asq-containing Peptide Libraries **22**–**27**. Reagents and coupling sequences are depicted in Supplementary Information:

The degradation of biologically active peptides by digestive enzymes represents a significant limitation to the development of peptide drugs. The incorporation of an amino acid isostere into biologically active peptides has emerged as an effective strategy for increasing the metabolic stability of peptides, and it was envisaged that the use of the α-Asq moiety in this regard would enhance the stability of the resulting peptides towards digestive enzymes. To evaluate the feasibility of this strategy, we developed a model study using peptide **28** and carboxypeptidase Y (Figure 2). The α-Asq-containing peptide **28** was prepared using solid phase synthesis (Scheme SI-3) and subsequently subjected to an enzymatic hydrolysis reaction using carboxypeptidase Y. The time course of the enzymatic hydrolysis reaction was monitored by MALDI-TOF MS (Figure 2). After 2 hours, all of the starting peptide was converted to to peptide **29** [*m/z* 880], where the three amino acid residues -Ser-Tyr-Phe-OH had been cleaved from the C-terminal of **28** (Figure 2, (c)). These results indicated that the α-Asq moiety was stable to carboxypeptidase Y. The α-Asq-containing peptide libraries was employed to random screening using two assay sistems: (i) rat hepatoma cells (dRLh-8) proriferation effects and (ii) inihibitory effects toward acetylcholinesterase (see SI). Among them, peptide library **23** exhibited moderate inhibitory activity towards dRLh8 cancer cells at 100 μM whereas the other libraries appeared to be inactive at this

concentration. None of the libraries exhibited inhibitory activity towards acetylcholinesterase at a dose of 100 μg/mL.

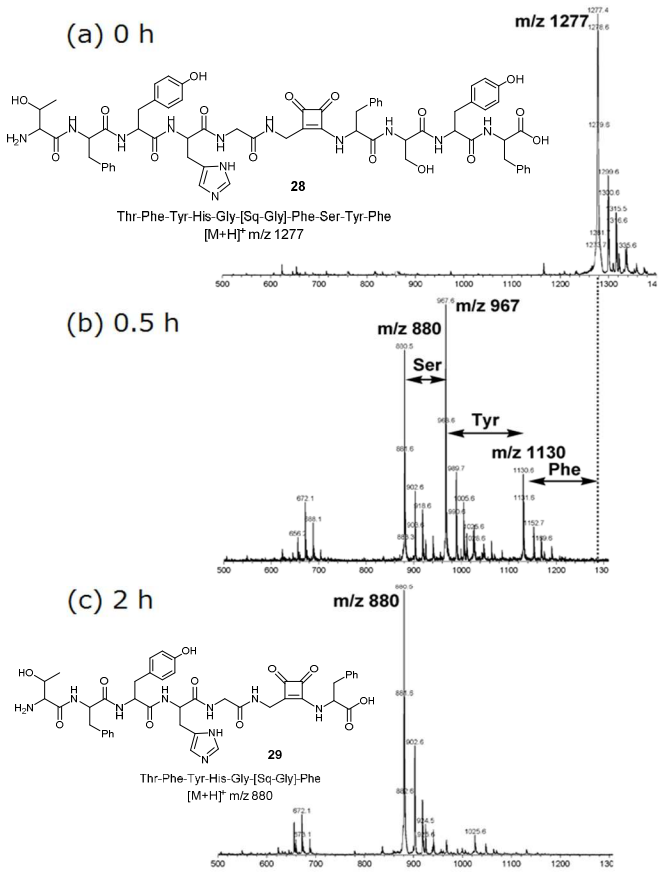


Figure 2. Enzymatic hydrolysis reaction of peptide **28** with carboxypeptidase Y. MALDI-TOF MS spectra of the crude mixture at (a) 0 h, (b) 0.5 h, (c) 2 h. Matrix: α-CHCA, results recorded in the positive ion mode.

In summary, we have developed a method for the solid phase synthesis of α-Asq-containing peptides and peptide libraries. We examined the effect of α-Asq toward protease usig carboxypeptidase Y for the first time. The α-Asq moiety was found to be resistant to carboxypeptidase Y. This results woul aid to the desing and synthesis of stable α-Asq incorporating peptides. The biological screening of the libraries generated in this study revealed that **23** exhibited moderate inhibitory activity towards dRLh-8 cells at 100 μM. Further library screening studies, and studies directed towards the application of novel peptide analogues in chemical biology,[6] are currently underway in our laboratory.

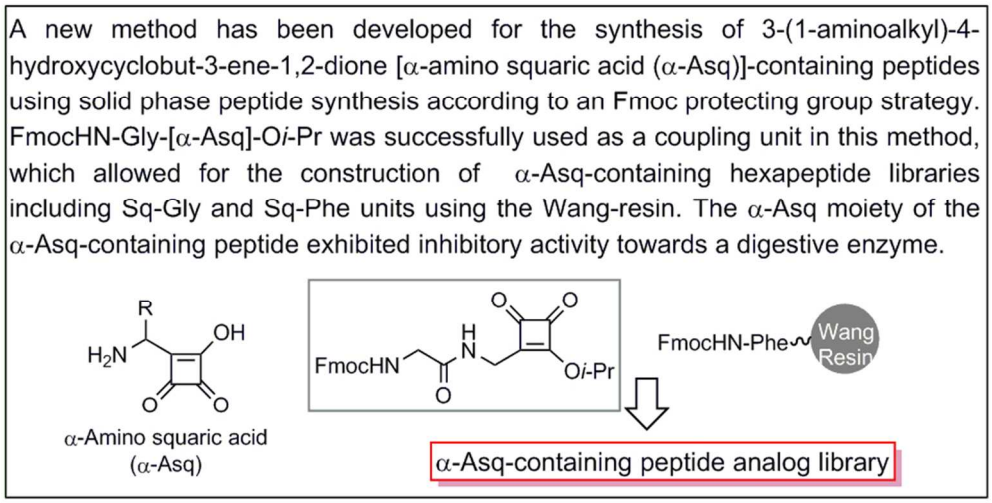
Notes and references

[a] Graduate School of Science, Osaka City University, 3-3-138, Sugimoto, Sumiyoshi, Osaka 558-8585, Japan
[b] Office of Research Exchange, Iwate University, 3-18-8 Ueda, Morioka, Iwate 020-8550, Japan
[c] Faculty of Pharmacy, Kinki University, 3-4-1 Kowakae, Higashiosaka, Osaka 577-8502, Japan

† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/c000000x/

Acknowledgments
This work was financially supported by grants from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) (No. 23102009), and the Japan Society for the Promotion of Science (KAKENHI, Grant Nos. 23228001 & 25282233).

1. (a) M. Gongora-Benitez, J. Tulla-Puche and F. Albericio, *ACS Combinatorial Science,* 2013, **15**, 217; (b) *Amino Acids, Peptides and Proteins in Organic Chemistry, Protection Reactions, Medicinal Chemistry, Combinatorial Synthesis, Vol. 4*, A. B. Hughes, ed.; WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim, 2011; (c) Peptides: Chemistry and Biology, 2nd Ed., N. Sewald and H.-D. Jakubke, eds.; WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim, 2009.
2. (a) O. Melnyk, N. Ollivier, S. Besret and P. Melnyk, *Bioconjugate Chem.* 2014, **25**, in press; (b) L. Kiss and F. Fülöp, *Chem. Rev.* 2014, **114**, 1116; (c) Y. Ishibashi and M. Kitamura, *Chem. Commun.* 2009, **45**, 6985; (d) P. Cudic and M. Stawikowski, Peptidomimetics: Fmoc Solid-Phase Pseudopeptide Synthesis; In *Peptide-Based Drug Design*, Otvos, L. ed.; Humana Press, 2008, 223; (e) N. G. Angelo and P. S. Arora, *J. Am. Chem. Soc.*, 2005, **127**, 17134.
3. For reviews: (a) F. R. Wurm and H.-A. Klok, *Chem. Soc. Rev.,* 2013,**42**, 8220; (b) C. Ballatore, D. M. Huryn and A. B. Smith, III, *ChemMedChem.* 2013, **8**, 385; (c) C. M. Marson, *Chem. Soc. Rev.,* 2011, **40**, 5514; (d) R. I. Storer, C. Aciro and L. H. Jones, *Chem. Soc. Rev.*, 2011, **40**, 2330; (e) T. Shinada, T. Ishida and Y. Ohfune, *J. Syn. Org. Chem., Jpn.*, 2007, **65**, 32; (f) M. Ohno and S. Eguchi, In *Bioactive Heterocycles I*: *Topics in Heterocyclic Chemistry*; S. Eguchi, ed.; Vol. 6; Springer: Berlin, 2006; pp 1–37; (g) G. Seitz and P. Imming, *Chem. Rev.,* 1992, **92**, 1227; (h) F. Serratosa, *Acc. Chem. Res.,* 1983, **16**, 170; (i) A. H. Schmidt, *Synthesis,* 1980, 961. (j) R. West, In *Oxocarbons*; Academic: New York, 1980. (k) R. West and J. Niu, In *Nonbenzenoid Aromatics*; J. P. Snyder, Ed.; Academic: New York, 1969; p 311.
4. (a) T. Shinada, A. Yamasaki, Y. Kiniwa, K. Shimamoto and Y. Ohfune, *Tetrahedron Lett.,* 2009, **50**, 4354; (b) T. Shinada, T. Ishida, K. Hayashi, Y. Yoshida, Y. Shigeri and Y. Ohfune, *Tetrahedron Lett.,* 2007, **48**, 7614; (c) T. Ishida, T. Shinada and Y. Ohfune, *Synthesis* 2005, 2723; (d) T. Ishida, T. Shinada and Y. Ohfune, *Tetrahedron Lett.,* 2005, **46**, 311; (e) T. Shinada, K.Hayashi, T. Hayashi, Y. Yoshida, M. Horikawa, K. Shimamoto, Y. Shigeri, N. Yumoto and Y. Ohfune, *Org. Lett.,* 1999, **1**, 1663.
5. "Compound **15** was labile under the coupling reaction conditions (basic conditions) to give a complex mixture due provably to the presence of a reactive active methine in the molecule. On the other hand, compound **20** is more stable due to the protecting effect of the active methine with a benzyl group.".
6. (a) S. Ziegler, V. Pries, C. Hedberg and H. Waldmann, *Angew. Chem. Int. Ed.,* 2013, **52**, 2744; (b) M. Ueda, *Chem. Lett.,* 2012, **41**, 658.



81x41mm (300 x 300 DPI)