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A Strategy To Prepare Peptide Heterodimers in the Solid Phase with an Acid-Labile Linker

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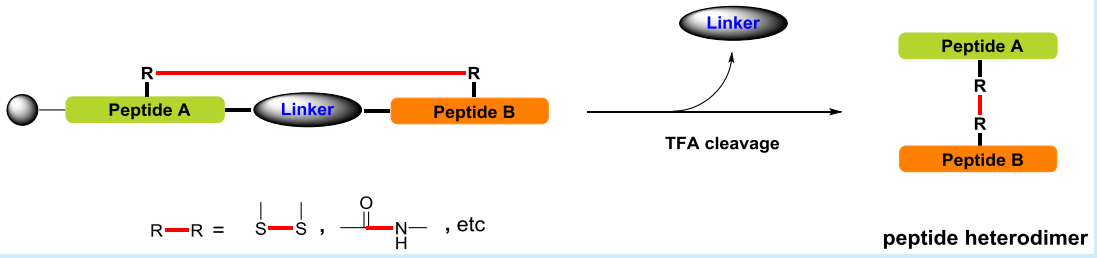
Supporting Information



ABSTRACT: The currently synthetic methods of peptide heterodimer involve tedious synthesis and purification steps. An acid-labile traceless linker was prepared, which is highly compatible with the Fmoc strategy and could be used to prepare peptide heterodimer on resin. The linker could be cleaved concomitantly with peptide cleavage, and two model heterodimers were synthesized. The proposed synthesis procedure is simple, straightforward, and provides great convenience for preparing disulfide-linked peptide heterodimers.

Various peptides and small proteins, such as insulin and relaxin, exist as heterodimers, which are attractive targets for the development of next-generation therapeutics owing to their pleiotropic physiological properties.[1,2] Research on the structure−function relationship in heterodimeric peptides plays an important role in developing new lead compounds,[3,4] and finding effective and selective approaches to achieve heterodisulfide formation between two peptide chains is an important area of research.

Several methods to prepare heterodisulfide peptide dimers have been published. The initial method involved the separate assembly of two S-reduced chains and subsequent oxidative folding in solution. However, this method produced randomly formed heterodimers, which means the target product was not always obtained. Thus, this method was limited in scope.[5,6]

Another important strategy to prepare heterodisulfide peptide dimers involved the directed, stepwise formation of disulfide bridges. This method activated the thiol group of one peptide and then added the second peptide in its free thiol form.[3,7,8] Here, the target asymmetric heterodimer was formed via thiol exchange in acidic conditions without formation of homodimer. However, the two peptides must be prepared and purified before they can be combined, which involves tedious steps.[9] This method also required orthogonal S-protection and the solid-phase synthesis of the two separate chains followed by their release from the solid support. Multistep solution-phase reactions can produce the correct disulfide pairing but result in low yields.[10,11] Thus, while this method was very effective, its complexity emphasized the need for an improved synthetic route to obtain heterodimeric peptides through fewer synthetic steps.

Patil[12] reported a method that imitated the insulin synthesis procedure in vivo cellular, whereby both chains are sequentially synthesized on the same solid support and separated by a chemically cleavable bis-linker tether. In this method, the two peptide chains were conveniently synthesized, the disulfide bridge was formed in solution, and the bis-linker tether was removed with hydrazine buffer. However, this strategy also presented several obvious drawbacks. First, 4-(hydroxymethyl)benzamide in the tether linker[13] is base labile and prone to nucleophilic attack. More importantly, in this method, the disulfide bonds are formed in solution. To ensure the intramolecular disulfide formation, oxidation was performed in very dilute solution. These processes render the workup tedious.

In the present work, we develop an analogous strategy that is simple, compatible, and straightforward for overcoming the limitations described above and develop a facile synthesis procedure. Our proposed procedure involves an acid-labile linker and allows intramolecular coupling on resin. Two key structures are essential to link two peptide chains together: one

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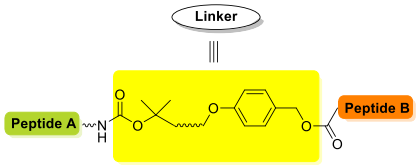
to anchor the N-terminal and another to anchor the C-terminal. As *tert*-butyloxycarbonyl (Boc) and *p*-alkoxybenzyl benzyl (linker of Wang resin) are widely used in peptide synthesis and labile to acidic conditions, we synthesized a linker containing these molecules. Similar to the Boc and *p*-alkoxybenzyl alcohol, the designed linker is compatible with the Fmoc-tBu SPPS strategy and most disulfide bridge-formation conditions and also easily removed during peptide acidic cleavage (Scheme 1).

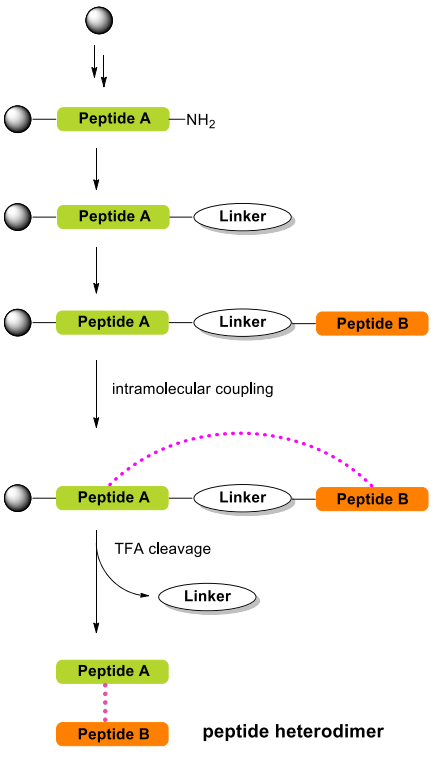
Scheme 1. Structure of Acid-Labile Linker



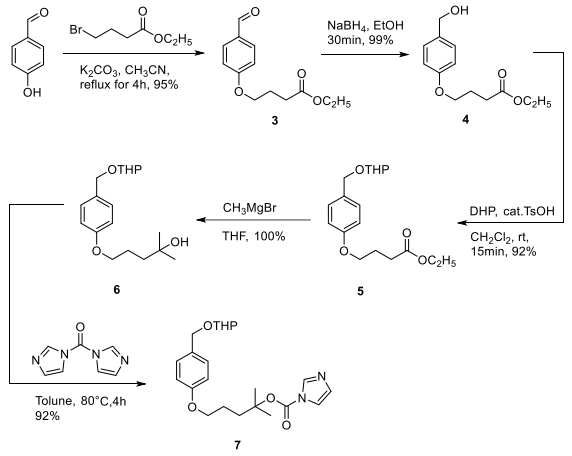
To obtain a heterodimer, peptide **A** was first assembled via the standard Fmoc-SPPS strategy with an acid-labile resin, such as Wang resin. The tether linker was then anchored to the free α-NH_2 group, and the free OH was exposed. The C-terminal amino acid of peptide B was subsequently anchored to the OH group. Standard SPPS amino acid coupling was carried out to complete the assembly of peptide B. The intramolecular disulfide bond was next formed on resin as shown in Scheme 2.

The linker is easy to prepare in high yield (Scheme 3). To construct the molecular bridge, 4-hydroxybenzaldehyde was used as the initial material. 4-Hydroxybenzaldehyde was reacted with a halogen aliphatic carboxyl ester that is used to form the Boc-like structure in the next step. To decrease the

Scheme 2. Strategy for Preparing Peptide Heterodimers in the Solid Phase via an Acid-Labile Linker



Scheme 3. General Synthesis Procedure of Acid-Labile Linker



effect of the benzene ring, a suitable length is necessary; here, we used ethyl 4-bromobutyrate to give compound **3**. The ester terminal was transferred to tertiary alcohol **6** by reaction with excess Grignard reagent CH_3MgBr. Before reacting, the OH groups in **4** were preprotected with DHP (3,4-2*H*-dihydropyran) to give compound **5**. The tertiary alcohol in **6** was then activated to react with the free α-amino groups. Compound **6** was first considered to transfer to the corresponding *p*-nitrophenyl carbonate, but the transformation encounter failed. Finally, we found that imidazole carbonate **7** prepared from tertiary alcohol **6** and carbonyl diimidazole could be obtained in excellent yield. Surprisingly, **7** is quite stable and easy to store.

Using the proposed method, various heterodimers could be easily prepared, regardless of the bridge types (i.e., disulfide or lactam). In the present case, we only demonstrate how to prepare a disulfide-bridge type peptide heterodimer. To illustrate the overall method, a short insulin fragment was synthesized as a model by using polystyrene-divinylbenzene Wang resin (0.56 mmol/g) as the solid support (Scheme 4) and Fmoc-Cys (Acm)-OH as the Cys residue. The first step involves the sequential assembly of chain A via the standard Fmoc-SPPS procedure to give peptide resin **9a**. Linker **7** was anchored to the free α-NH_2 of **9a** to give peptide resin **9b** (Scheme 5). To anchor linker **7** to the α-NH_2 in **9a**, we first dissolved **7** and TEA in CH_2Cl_2 with DMAP as the catalyst and then allowed the solution to react with peptide resin **9a** for 24 h. The Kaiser test gave a positive result, which means **7** either reacts with amino groups with a slow rate or does not react at all. Other methods, such as DBU[14] catalysis, also yielded a negative result. Hence, **7** was transferred to an onium salt to enhance its activity. CH_3I[15] and TfOMe[16,17] have been reported to be suitable reagents for this purpose; here, we used TfOMe as an additive to transfer **7** to onium salt **8**, which reacts completely and immediately. The high reactivity of **8** could acylate free amino groups in **9a** smoothly to give the peptide resin **9b** (Scheme 5). Onium salt **8**, however, is not stable, and its slow and continuous decomposition in solution could observed. Hence, the onium salt solution should be used immediately after preparation.

Next, the THP group of **9b** was removed, and elongation was continued through the OH group of the linker. Since TsOH has been used as a deprotecting reagent for THP during solid-phase peptide synthesis,[18] this reagent was selected to

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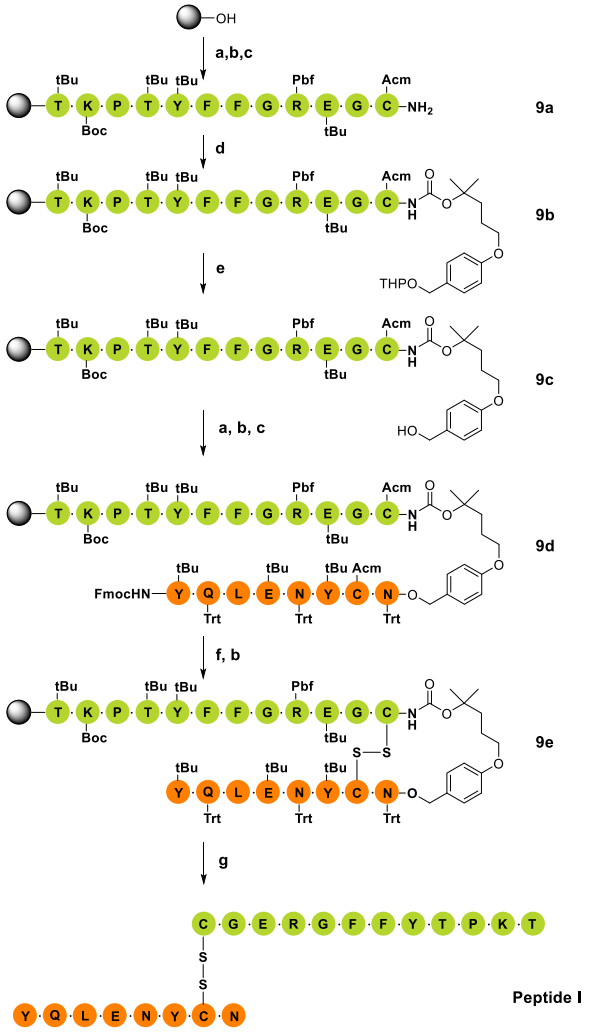
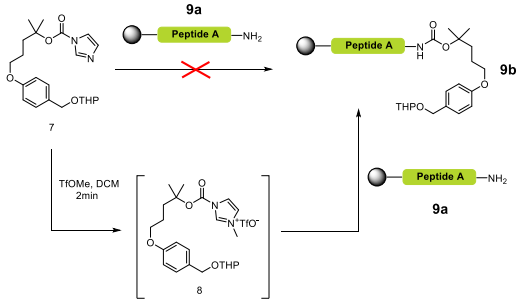
Scheme 4. Preparation of Insulin Fragments Containing One Intermolecular Disulfide Bridge[a]



[a]Reagents and conditions: (a) 10 equiv of Fmoc-AA-OH, 5 equiv of DIC, 0.2 equiv of DMAP in CH_2Cl_2-DMF (9:1, v/v), 8 h; (b) 20% piperidine−DMF, 15 min; (c) 4 equiv of Fmoc-AA-OH, 3.8 equiv of HATU, 8 equiv of DIPEA in DMF, 2 h; (d) 8 equiv of **8**, 10 equiv of Et_3N in anhydrous CH_2Cl_2, 12 h × 2; (e) 5 mg/mL of TsOH in CH_2Cl_2−MeOH (9:1, v/v), 60 min × 2; (f) 5 equiv of I_2 in DMF, 15 min × 2; (g) TFA/TIS/EDT/*m*-cresol/H_2O (90:2.5:2.5:2.5:2.5, v/v), 1 h.

Scheme 5. Loading of Linker 7 to the Peptide Resin 9a



remove OH groups in this work. A mixture of *p*-TsOH (5 mg/mL) in CH_2Cl_2−MeOH (9:1, v/v) was found to complete the deprotection within 60 min × 2, giving peptide resin **9c**. Note that the TsOH solution does not affect commonly used acid-labile side chain-protecting groups, but Cys (Trt), Cys (Mmt), and His (Trt) were found to deprotect slowly when treated with TsOH, so these amino acid residues were recommended for use in chain B.

The next procedure involves an esterification of the free OH in linker. Similar to the common loading situation, the DIC-DMAP method is effective for most common amino acids, but for the Cys and His situation, the MSNT/MeIm method was chosen to decrease the epimerization.[19] As a benefit of the the high reactivity of the acylation, the esterification was complete with 10 equiv of Fmoc amino acid within 8 h. The completion of esterification and THP deprotection was confirmed by the Fmoc quantification loading test.[19] Chain B was sequentially assembled to give **9d**. A small amount of **9d** was subjected to Fmoc removal, cleaved, and then analyzed by RP-HPLC. Two sharp peaks with good purity were observed in Figure 1a,

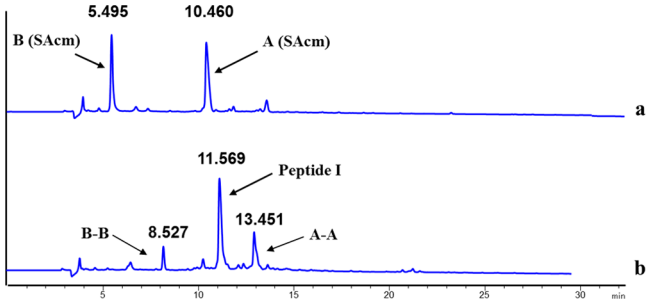


Figure 1. RP-HPLC chromatograms data: (a) mixture of chain A and chain B; (b) crude peptide I. CH_3CN−H_2O (0.1% TFA) as the eluent system (1 mL/min, 20%−50%, 30 min).

which indicates that the acid-labile linker was removed and that the two peptide chains were synthesized successfully. The disulfide bond was formed on resin by using I_2 in DMF[20] for 15 min × 2. Finally, the peptide was cleaved by TFA/TIS/EDT/*m*-cresol/H_2O (90:2.5:2.5:2.5:2.5, v/v) solution. After concomitant cleavage of the molecular bridge, the peptide heterodimer was obtained. Crude peptide I was analyzed by RP-HPLC, and a sharp peak was observed (11.6 min in Figure 1b). Two byproducts (8.5 and 13.5 min in Figure 1b) were identified as two homodimers. This result was confirmed by MS and reveals that the peptide heterodimer was successfully obtained. The results also show that intermolecular coupling is inevitable in the preparation of the intramolecular disulfide bond by oxidation.

The proposed strategy may also be employed to develop more complicated heterodimers, which contains two disulfide bonds. TD1 (ACSSSPSKHCG) is a transdermal peptide that has been shown to facilitate efficient transdermal protein drug delivery through intact skin.[21] Oxytocin (OXT, CY-IQNCPLG) is a peptide hormone and neuropeptide that is known to affect a variety of social and nonsocial activities and behaviors.[22] Herein, we prepared a TD1-OXT (OXT as an analogue with carboxyl acid in C-terminal) heterodimer by using two intermolecular disulfide bridges via our method to illusion the two-disulfide situation chemically (Scheme 6).

In order to show the compatibility of the traceless linker and avoid the formation of homodimers, we considered the nonoxidative solid-phase method to prepare the heterodimer TD1-OXT, which was mediated by 2,2′-dithiobis(5-nitropyridine) (DTNP).[11,23] The OXT analogue was first

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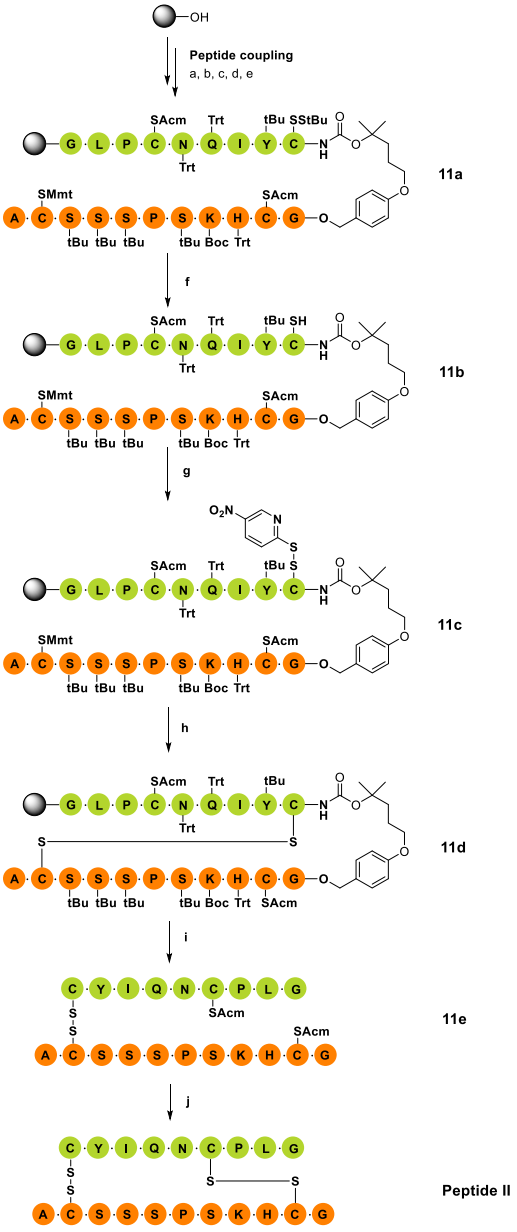
Scheme 6. Preparation of TD1-OXT Peptide Containing Two Intermolecular Disulfide Bridges[a]



[a]Reagents and conditions: (a) 10 equiv of Fmoc-AA-OH, 5 equiv of DIC, 0.2 equiv of DMAP in CH_2Cl_2–DMF (9:1, v/v), 8 h; (b) 20% piperidine–DMF, 15 min; (c) 4 equiv of Fmoc-AA-OH, 3.8 equiv of HATU, 8 equiv of DIPEA in DMF, 2 h; (d) 8 equiv of **8**, 10 equiv Et_3N in anhydrous CH_2Cl_2, 12 h × 2; (e) 5 mg/mL of TsOH in CH_2Cl_2–MeOH (9:1, v/v), 60 min ×2; (f) 20% BME in DMF, 0.1 M DIPEA, 10 h; (g) 10 equiv of DTNP in CH_2Cl_2, 1 h; (h) TFA/TIS/CH_2Cl_2 (1:5:94, v/v), 6 × 3 min, then in DMF for 1 h; (i) TFA/TIS/EDT/*m*-cresol/H_2O (90:2.5:2.5:2.5:2.5, v/v), 1 h; j) I_2 in HOAc–H_2O (4:1, v/v).

assembled as chain A and TD1 as chain B. To prepare the heterodimer peptide, Cys should be orthogonally protected and the disulfide bonds precisely matched. StBu-Acm was used to protect chain A, while Mmt-Acm was used to protect chain B. The disulfide bond close to the N-terminals of A and B was considered to form first to avoid the steric hindrance of the solid phase. The StBu group in chain A was removed by treating the peptide-resin with 0.1 M DIPEA in 20% BME/DMF for 10 h. The resulting resin **11b** was reacted with 10 equiv of DTNP for 1 h, and the free thiol group was reprotected with 5-nitropyridinsufenyl (5-Npys) to give resin **11c**. Resin **11c** was then treated with dilute TFA solution (TFA/TIS/CH_2Cl_2 = 1:5:94, v/v) for 6 × 3 min to remove the Mmt group in chain B. Thereafter, the 5-Npys group was protonated and activated to attack the free thiol group. The first disulfide bond was completed after further agitation in DMF for 1 h. The peptide was then cleaved by TFA solution. The resulting crude peptide was subsequently purified and analyzed by RP-HPLC (Figure 2). The purified **11e** was then diluted with acetic acid prior to treatment with excess iodine (I_2) to form the second disulfide (Figure 3).

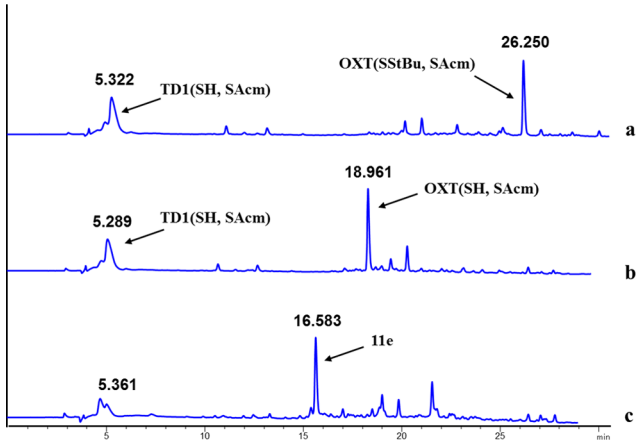


Figure 2. RP-HPLC chromatograms data: (a) microcleavage of peptide–resin **11a** and analysis; (b) microcleavage of peptide–resin **11b** and analysis; (c) crude **11e**. CH_3CN–H_2O (0.1% TFA) as the eluent system (1 mL/min, 10%–40%, 30 min).

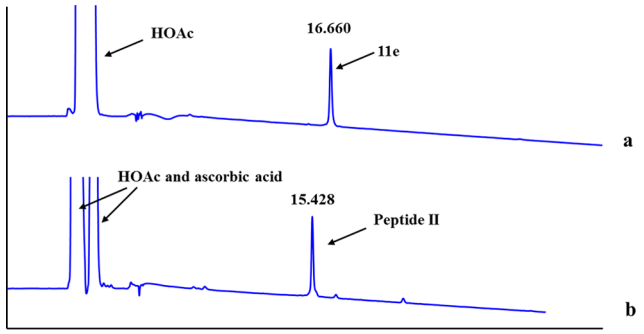


Figure 3. RP-HPLC chromatograms data during I_2 oxidation: (a) purified **11e** (dissolved in HOAc–H_2O = 4:1, v/v); (b) peptide II. CH_3CN–H_2O (0.1% TFA) as the eluent system (1 mL/min, 10%–40%, 30 min).

In conclusion, we offer a new approach to prepare peptide heterodimers containing intermolecular disulfide bridges with the assistance of an acid-labile linker. Both oxidation and nonoxidation methods can match the strategy effectively. The proposed method is highly compatible with the Fmoc strategy and, therefore, could be used in many applications. More importantly, the linker is easily prepared and extremely stable. Using our method, two heterodimers were synthesized, and intermolecular disulfide bonds between the two peptide chains were formed on resin in one pot, thereby minimizing the need for tedious synthesis and purification steps.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.9b02638.

Detailed experimental procedures, characterizations, and spectroscopic data (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Teichman, S. L.; Unemori, E.; Dschietzig, T.; Conrad, K.; Voors, A. A.; Teerlink, J. R.; Felker, M.; Metra, M.; Cotter, G. *Heart Failure Rev.* **2009**, *14*, 321−329.
(2) Karas, J.; Shabanpoor, F.; Hossain, M. A.; Gardiner, J.; Separovic, F.; Wade, J. D.; Scanlon, D. B. *Int. J. Pept.* **2013**, *2013*, 1−8.
(3) Galande, A. K.; Spatola, A. F. *Org. Lett.* **2003**, *5* (19), 3431−3434.
(4) Mullen, D. G.; Verardi, R.; Porcelli, F.; Scaloni, A.; Barany, G.; Veglia, G. *Biopolymers* **2012**, *98* (5), 479−484.
(5) Dekan, Z.; Headey, S. J.; Scanlon, M.; Baldo, B. A.; Lee, T. H.; Aguilar, M. I.; Deuis, J. R.; Vetter, I.; Elliott, A. G.; Amado, M.; Cooper, M.; Alewood, D.; Alewood, P. *Angew. Chem., Int. Ed.* **2017**, *56* (29), 8495−8499.
(6) Dalla Serra, M.; Cirioni, O.; Vitale, R. M.; Renzone, G.; Coraiola, M.; Giacometti, A.; Potrich, C.; Baroni, E.; Guella, G.; Sanseverino, M.; De Luca, S.; Scalise, G.; Amodeo, P.; Scaloni, A. *Biochemistry* **2008**, *47* (30), 7888−7899.
(7) Vila-Perelló, M.; Tognon, S.; Sánchez-Vallet, A.; García-Olmedo, F.; Molina, A.; Andreu, D. *J. Med. Chem.* **2006**, *49* (2), 448−451.
(8) Chen, L.; Golser, R.; Machová, A.; Slaninová, J.; Barany, G. *J. Med. Chem.* **1999**, *42* (24), 5002−5009.
(9) Zaykov, A. N.; Gelfanov, V. M.; Liu, F.; Dimarchi, R. D. *Org. Lett.* **2018**, *20*, 3695−3699.
(10) Disotuar, M. M.; Petersen, M. E.; Nogueira, J. M.; Kay, M. S.; Chou, D. H.-C. *Org. Biomol. Chem.* **2019**, *17*, 1−12.
(11) Wu, F.; Mayer, J. P.; Gelfanov, V. M.; Liu, F.; Dimarchi, R. D. *J. Org. Chem.* **2017**, *82* (7), 3506−3512.
(12) Patil, N. A.; Tailhades, J.; Karas, J. A.; Separovic, F.; Wade, J. D.; Hossain, M. A. *Angew. Chem., Int. Ed.* **2016**, *55* (47), 14552−14556.
(13) Hansen, J.; Diness, F.; Meldal, M. *Org. Biomol. Chem.* **2016**, *14* (12), 3238−3245.
(14) Larrivée-Aboussafy, C.; Jones, B. P.; Price, K. E.; Hardink, M. A.; McLaughlin, R. W.; Lillie, B. M.; Hawkins, J. M.; Vaidyanathan, R. *Org. Lett.* **2010**, *12* (2), 324−327.
(15) Vaillard, V. A.; Gonzalez, M.; Perotti, J. P.; Grau, R. J. A.; Vaillard, S. E. *RSC Adv.* **2014**, *4*, 13012−13017.
(16) Wasa, M.; Engle, K. M.; Lin, D. W.; Yoo, E. J.; Yu, J. *J. Am. Chem. Soc.* **2011**, *133* (133), 19598−19601.
(17) Roychoudhury, R.; Pohl, N. L. B. *Org. Lett.* **2014**, *16* (4), 1156−1159.
(18) Kuisle, O.; Quiñoá, E.; Riguera, R. J. *J. Org. Chem.* **1999**, *64* (22), 8063−8075.
(19) Jensen, K. J.; Shelton, P. T.; Pedersen, S. L. *Peptide Synthesis and Applications*, Second.; 2013.
(20) Tulla-puche, J.; Nú ria Bayó-Puxan; Moreno, J. A.; Francesch, M.; Cuevas, C.; AÄ lvarez, M.; Albericio, F. *J. Am. Chem. Soc.* **2007**, *129*, 5322−5323.
(21) Wen, L. P.; Ma, M.; Zhang, C.; Shen, Y.; Chen, Y.; Zhang, M.; Guo, X.; Yang, W.; Liu, S. *Nat. Biotechnol.* **2006**, *24* (4), 455−460.
(22) Yang, H.; Wang, L.; Han, L.; Wang, S. C. *ISRN Neurosci.* **2013**, 1−13.
(23) Galande, A. K.; Weissleder, R.; Tung, C.-H. *J. Comb. Chem.* **2005**, *7* (2), 174−177.

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