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Total Synthesis of Human Galanin-Like Peptide through an Aspartic Acid Ligation

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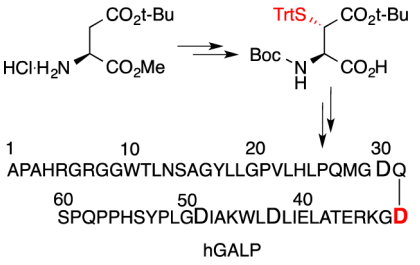
ABSTRACT



Human galanin-like peptide (hGALP) is a newly discovered hypothalamic peptide that plays important roles in the regulation of food intake and energy balance. Here, we demonstrate that the aspartic acid ligation can be employed to achieve an efficient synthesis of hGALP. The total synthesis of hGALP enhances our ability to study its biology and facilitates the development of more stable analogues.

Chemical synthesis is a valuable tool for studying the structure and function of naturally occurring proteins as well as modified and engineered proteins because it imparts a previously unattainable level of control over protein composition.[1] Encouraged by previous studies on protein chemical synthesis, we initiated a program directed toward the total chemical synthesis of a newly discovered neuropeptide, human galanin-like peptide (hGALP).[2] Recent findings have revealed that GALP plays important roles in energy metabolism, thermoregulation, reproduction, as well as appetite control.[3] When administered by intracerebroventricular (i.c.v.) injection, GALP decreases overall food intake and causes a decrease in body weight in rats.[4] Because of its important physiological functions, there is a strong interest in developing GALP as a therapeutic for the treatment of obesity.[3] However, similar to other peptide regulators, GALP has a very short half-life in blood, which necessitates frequent injections or infusions.[5] Therefore, the development of metabolically more stable analogues of GALP is needed. Chemical synthesis could be well suited for this purpose. As has been widely demonstrated, once a general synthetic route to GALP is established, its synthetic variants can be produced relatively quickly. In principle, GALP analogues with more suitable bioprofiles can be identified by screening the collection of its synthetic variants.

The synthesis of hGALP (**1**) may be achieved in several ways. Detailed analysis of its sequence suggested to us the possibility of disconnecting it into two fragments (1−31) **3**

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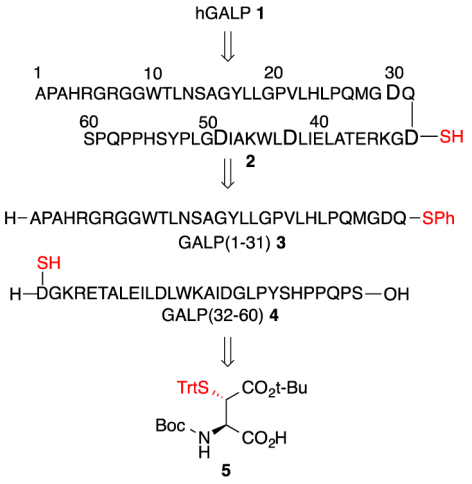
Scheme 1. Retrosynthetic Analysis for the Synthesis of hGALP

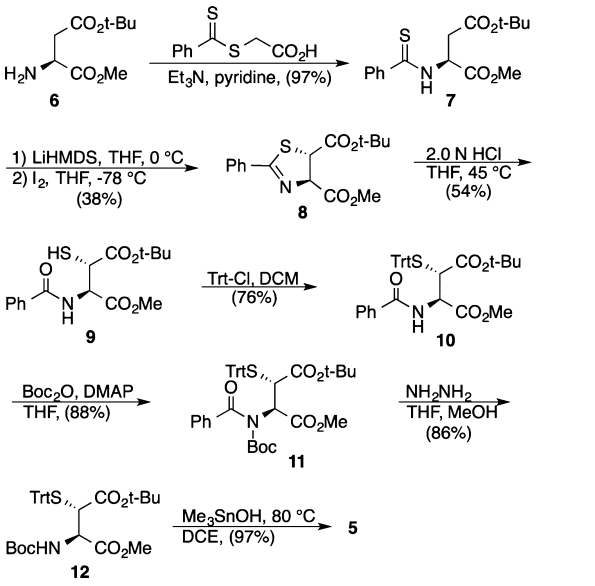


and (32−60) **4**, each containing about 30 amino acids. In this way, it may be possible to effectively prevent synthetic problems that may arise from aspartimide formation.[6] Human GALP contains four Asp residues (Scheme 1, enlarged amino acids). By strategically disconnecting hGALP at the Gln-Asp site, two aspartic acids, Asp30 and Asp32, are the first and last building block being added to the growing peptide chain during Fmoc-based solid-phase peptide synthesis (SPPS). They are either not next to a α-carboxamide or not exposed to strong bases and are thus stable during synthesis. Aspartimide formation at Asp43 and 49 can be reduced by using Fmoc-Asp(OMpe)-OH and Fmoc-Asp(OtBu)-(Dmb)Gly-OH (Mpe = β-3-methylpent-3-yl, Dmb = 2,4-dimethoxybenzyl).[6b,c]

We began our synthesis by preparing β-thioaspartic acid **5**. As shown in Scheme 1, the Asp surrogate **5** has two functional groups, a sulfhydryl group and a carboxyl group, attached to its β-carbon atom. There are two possible diastereomers of protected thioaspartic acids: **5** and its β-epimer.[7] Recently, Payne and co-workers reported a native chemical ligation at aspartic acid. Their results showed that there is no noticeable difference in the ligation rate between the two epimers.[7d]

To develop an efficient synthesis of β-thioaspartic acid **5**, we attempted the direct thiylation of the protected aspartic acid (Scheme 2). Previous work on stereoselective synthesis of *trans*-oxazoline via iodination of dianion marginally supports the feasibility of such a synthetic strategy.[8] To test

Scheme 2. Synthesis of the Ligation Precursor of Aspartic Acid[a]



this hypothesis, we first prepared the thiobenzamidomalonic ester **7**.[9] The lithium dianion of **7** was formed at 0 °C in THF using LiHMDS (2.0 equiv). Happily, quenching the dianion with I_2 followed by an acidic workup gave the *trans* thiazoline **8** in 38% yield over two steps.[8] No *cis*-isomer was detected. In addition to the desired product, about 30% 1,3-thiazole compound was also produced in this reaction. Selective hydrolysis of thiazoline **8** using 2.0 N HCl afforded the *N*-benzoyl derivative **9** in 54% yield.[8] The free thiol in **9** was protected with a trityl group. To decrease the radical-mediated addition of the thiol to the aromatic carbons, this reaction was carried out in the absence of base.[10] After the trityl protection of the SH group, the benzoyl group was replaced by a Boc group under the optimized, nonepimerizing conditions.[11] Finally, the methyl ester deprotection was accomplished under mild conditions using trimethyltin hydroxide to give the desired aspartic acid surrogate **5** in 97% yield.[12] This new route is four steps longer than the method reported by Payne et al.[7d]

With the thioaspartic acid in hand, we went on to evaluate the feasibility and versatility of the aspartic acid ligation using a model ligation test system, which has been widely used in the mechanistic study of native chemical ligation (Table 1).[13] This study is similar to that of Thompson et al. The only difference is that this study used different model peptides and a different diastereomer of the chiral thioaspartic acid. First, we tested the feasibility of β-thioaspartic acid mediated ligations with peptide

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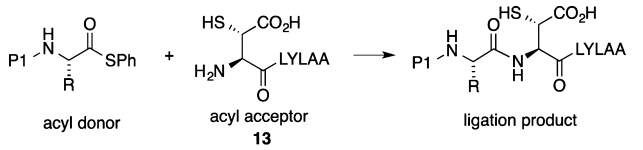
Table 1. Substrate Scope of Aspartic Acid Ligation[a]



	acyl donor	ligation product	yield [%]	*t* [h]
1	LYRA–Gly–SPh **14**	LYRA–Gly–LYLAA **15**	85	2
2	LYRA–Phe–SPh **16**	LYRA–Phe–LYLAA **17**	75	2
3	LYRA–Leu–SPh **18**	LYRA–Leu–LYLAA **19**	89	2
4	LYRA–Thr(OH)–SPh **20**	LYRA–Thr(OH)–LYLAA **21**	84	2
5	GKYRA–Val–SPh **22**	GKYRA–Val–LYLAA **23**	77	10
6	GKYRA–Ile–SPh **24**	GKYRA–Ile–LYLAA **25**	69	10

[a] Reagents and conditions: 6 M Gn·HCl, 300 mM Na_2HPO_4, 200 mM MPAA, 20 mM TCEP, pH 7.9. P1 = peptide fragments, Gn·HCl = guanidine hydrochloride, TCEP = tris(2-carboxyethyl)-phosphine, MPAA = 4-mercaptophenylacetic acid.

thioesters bearing C-terminal Gly (entry 1, Table 1). The reaction proceeded rapidly and was nearly completed in less than 10 min. After 2 h, the reaction was quenched with sodium 2-mercaptoethanesulfonate (MESNA). A subsequent HPLC purification gave the desired peptide **15** in an isolated yield of 85%. As expected, two other favorable thioesters **16** and **18**, which had C-terminal Phe and Leu residues, also underwent rapid ligation with the peptide **13** to generate the corresponding products **17** and **19** (entries 2 and 3). To further probe the scope of the aspartic acid ligation, peptide thioesters presenting β-branched C-terminal residues, including Thr, Val, and Ile, were also tested. Interestingly, the ligation reaction was not slowed by the β-hydroxyl group. It was also completed in 2 h to afford the desired peptide product in 84% isolated yield. As expected, the ligation reactions proceeded much less effectively with the Val and Ile peptide thioesters, **22** and **24**. As shown in Table 1, these reactions were nearly completed in 10 h to afford the ligation products **23** and **25** in relatively low yields (77% and 69%, respectively). The sequences of **22** and **24** were changed to prevent the overlap of the peaks of **23** and **25** and that of MPAA during HPLC purification. Similar to the ligations of isocysteine and γ-mercaptoglutamine, the rate and efficiency of aspartic acid ligation was not found to be markedly different from Cys-mediated reaction, which indicates that the presence of a neighboring carboxyl group does not significantly affect the ability of the β-thiol to catalyze native chemical ligations.[7d,14]

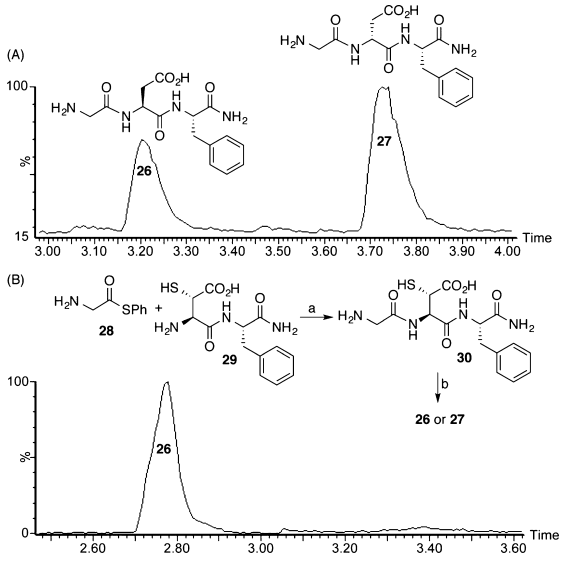


Figure 1. (A) LC–MS trace of the authentic tripeptides. (B) LC–MS trace of the desulfurization products. Reagents and conditions: (a) 6 M Gn·HCl, 300 mM Na_2HPO_4, 200 mM MPAA, 20 mM TCEP, pH 7.9. (b) TCEP, VA-044, *t*-BuSH, 1.5 h.

Having examined the reactivity of β-thioaspartic acid in ligation reactions, we next investigated the efficiency of the metal-free desulfurization (MFD) in converting thioaspartic acid into the natural aspartic acid. Happily, we found that the adjacent carbonyl group does not have a destructive effect on the desulfurization reaction. Under the original conditions, we were able to obtain all the desired peptide products in 4 h. The yield ranged from 50% for the desulfurization of **25** to 77% for that of **15**.[15]

We also confirmed that the stereochemical integrity of the α-carbon in the thioaspartic acid was preserved during all reaction steps. The level of racemization of aspartic acid was investigated based on the LC–MS analysis of a tripeptide, H-Gly-Asp-Phe-NH_2. We first prepared two authentic tripeptide samples, H-Gly-Asp-Phe-NH_2 **26** and H-Gly-DAsp-Phe-NH_2 **27**.[16] Consistent with previous reports, these two diastereomers can be well separated

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by LC−MS, exhibiting a difference in retention time of >0.5 min (Figure 1). The chirality of the thioaspartic acid can therefore be determined by comparing the desulfurization products of the tripeptide precursor **30** with **26** and **27**. The experiment was performed as shown in Figure 1B. After native chemical ligation of **28** and **29**, the roughly purified **30** was subjected to the standard MFD conditions. Only a trace amount of **27** ($<1\%$) was detected.

The effectiveness of aspartic acid ligation in the elimination of the problem of aspartimide formation was also investigated. The study was performed through the synthesis of peptide H-Leu-Tyr-Arg-Ala-Gly-Asp-Gly-Tyr-Leu-Ala-Ala-OH (**31**), which is highly prone to aspartimide formation. Under standard SPPS conditions, the synthesis using Fmoc-Asp(OtBu)-OH resulted in an extensive formation of inseparable aspartimide and related side products ($>50\%$). Although the use of Fmoc-Asp-(OMpe)-OH reduced the levels of aspartimide formation, the side reactions still occurred to the extent of approximately 10% in such a short peptide. As expected, the aspartic acid ligation provided sufficient protection against aspartimide formation. No detectable aspartimide products were observed during peptide synthesis, ligation, and desulfurization.

Having established the feasibility of Asp ligation for the preparation of aspartimide-prone peptide sequences, we set out to apply this strategy to the total synthesis of hGALP (**1**). Following the synthetic plan shown in Scheme 1, we first prepared the two hGALP fragments **3** and **4**. Similar to the synthesis of the model peptides, the peptide thioester **3** was prepared from the fully protected peptide fragment using the method described by Danishefsky and co-workers.[7c] The β-thioaspartic acid-containing peptide fragment **4** was synthesized by using **5**, Fmoc-Asp(OMpe)-OH, and Fmoc-Asp(OtBu)-(Dmb)-Gly-OH as building blocks during solid-phase synthesis. These two fragments were purified using HPLC. As expected, native chemical ligation between **3** and **4** proceeded rapidly under the standard conditions (6 M Gn·HCl, 300 mM Na_2HPO_4, 200 mM MPAA, 20 mM TCEP, pH 7.9) at room temperature. The reaction was completed in 2 h to afford the thiol-substituted hGALP **2** in a 56% isolated yield.

Having established the feasibility of Asp ligation for the preparation of **2**, we next attempted the MFD of **2**. As expected, the desulfurization reaction proceeded rapidly and cleanly [3 M Gn·HCl, 100 mM NaH_2PO_4, 250 mM TCEP, 40 mM glutathione, UV (365 nm), pH 6.5] to afford hGALP (**1**) in 80% isolated yield (Figure 2A).[15b] The LC−MS spectrum of the synthetic hGALP is identical to that of the commercial one, while its purity is higher than the reference sample. This result, together with the peptide sequencing with tandem MS/MS, confirmed the identity of synthetic product (Supporting Information). The CD spectrum of the synthetic hGALP also closely resembles that of the commercially available molecule, with a minimum at about 199 nm and a shape consistent with a random coil conformation (Figure 2B).

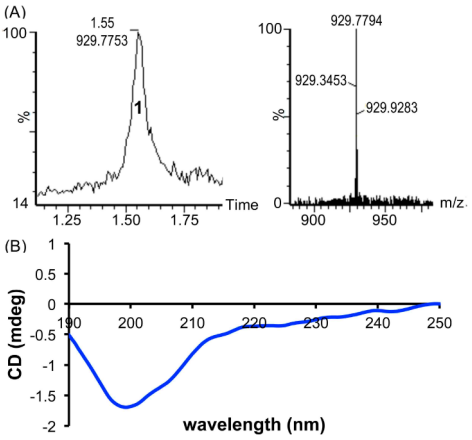

Figure 2. (A) LC−MS spectrum of the synthetic hGALP. $[M + 7H]^{7+}$ m/z = 929.0548 Da. (B) CD spectra of the synthetic hGALP in 5 mM phosphate buffer (pH 7) containing 100 mM NaF.

In summary, the study described here highlights the development of aspartic acid ligation for the synthesis of an attractive target, hGALP. To achieve this goal, we developed the synthesis of a useful native chemical ligation precursor, β-thioapartic acid. For the first time, we demonstrated the feasibility of direct thiylation for the enantioselective introduction of a thiol group to the β-carbon of amino acids. In addition, our studies revealed that the attachment of a carboxyl group on the adjacent carbon does not significantly change the reactivity of the thiol group in the NCL reaction. More importantly, we validated the efficacy of the Asp ligation in suppressing aspartimide formation. Finally, using the Asp-based NCL and MFD, we were able to synthesize hGALP with a high yield (44%) and purity. Our study further demonstrates the power of NCL/MFD in protein chemical synthesis and paves the way for future development of new hGALP analogues.

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Supporting Information Available. Experimental procedures; spectroscopic and analytical data. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.