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Total solid-phase synthesis of bombesin analogs with different functional groups at the *C*-terminus

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Abstract: Five bombesin analogs with different functional groups at the *C*-terminus were synthesized using a solid-phase strategy. The protocols were optimized using 4-(hydroxymethyl)benzoic acid (HMBA) resin to synthesize a common precursor followed by nucleophilic cleavage of the base sensitive peptide ester linkage. The *C*-terminal modifications included ethylamide, butylamide, methyl ester, propyl ester and hydrazide. Cleavage from the resin was possible with the fully protected or deprotected precursor peptide; however, higher purity of the final products was achieved when cleavage protocols were conducted after side-chain deprotection. The synthesized peptides were analyzed and characterized using reverse phase HPLC and ESI-MS. The peptides were obtained in 13–32% overall recovery, calculated from the coupling efficiency of the first amino acid residue, and in 91–97% purity. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: solid phase peptide synthesis; *C*-terminal modification

INTRODUCTION

Solid-phase peptide synthesis (SPPS), pioneered by Bruce Merrifield [1], has become an indispensable tool for peptide synthesis. In the ensuing years since its introduction, several attempts to improve and modify these synthetic methods have been reported. The large majority of these methods result in peptides that contain the carboxamide and to a lesser extent a carboxylic acid moiety [2–5]. Routes to peptides with different *C*-terminal functional groups have been recently reviewed [6,7]. Alsina and Albericio [6] have commented that a variety of Fmoc-compatible resins, including the 4-(hydroxymethyl)benzoic acid (HMBA) resin, have not yielded consistent results [6]. Recently, there has been a growing demand to synthesize peptides with various moieties at the *C*-terminus, to improve either their physicochemical or biological properties. One of the many peptides of longstanding interest in the scientific community is bombesin [8,9].

Bombesin and its mammalian counterpart gastrin-releasing peptide (GRP) have similar biological properties [10–12], and structural modifications within the *C*-terminal region can generate peptides that retain affinity without stimulating mitogenesis [13–20]. While these biological results have been published, only limited details of the syntheses have been reported [20] to our knowledge. Owing to the growing interest in the preparation of peptides with various chemical modifications at the *C*-terminus, we began a program to investigate these syntheses with Fmoc protected amino acid residues and the HMBA resin. In the present study we report the solid-phase synthesis and characterization of five potent *C*-terminal modified bombesin analogs (Table 1) obtained from nucleophilic cleavage of the base sensitive resin-handle of a common precursor (Figure 1).

MATERIAL AND METHODS

Chemicals and solvents were analytical reagent or HPLC grade. DCM, THF, methanol (MeOH) and TEA were purchased from Fisher Scientific, Fair Lawn, NJ, USA. Anhydrous $CaCl_2$ and TFA were purchased Mallinckrodt, Inc., St Louis, MO. DMF, Acetonitrile (MeCN), H_2O, DIPEA, *N*-methyl-2-pyrrolidone (NMP), piperidine, 1-propanol, hydrazine hydrate, methyl red, ethanol, *N*-propylamine, *N*-butylamine, trimethylorthoformate (TMOF) and ethylamine gas were purchased from Sigma Aldrich-St Louis, MO, USA. DIPCI, triisopropylsilane (TIS), diphenyldichlorosilane, 2,4,6-trinitrobenzenesulfonic acid (TNBSA), and thioanisole were purchased from Fluka Chemie GmbH, Buchs CH-9471, Switzerland. DMAP and 1,2-ethanedithiol (EDT) were purchased from Aldrich Chemical, Milwaukee, WI, USA. HBTU was purchased from Applied Biosystems, Warrington, UK. HOAt was purchased from Perseptive Biosystems, Framington, MA, USA. HOBt and HMBA resin were purchased from Novabiochem, La Jolla, CA, USA.

Fmoc-Leu-OH, Fmoc-Val-OH and Fmoc-Ala-OH were purchased from Novabiochem, La Jolla, CA, USA. Fmoc-His(Trt)-OH and Fmoc-Gln(Trt)-OH were purchased from Synthetech, Inc., Albany, OR, USA. Fmoc-D-Phe-OH was purchased from GL Biochem, Ltd., Shanghai, China. Fmoc-Gly-OH and Fmoc-Trp(Boc)-OH were purchased from Genzyme Pharmaceuticals, Cambridge, MA, USA.

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Table 1 Bombesin analogs with *C*-terminal modifications

Peptide	Amino acid sequence							
	6	7	8	9	10	11	12	13
Bombesin [6–13]; BBN [6–13]	Asn	Gln	Trp	Ala	Val	Gly	His	Leu-NH_2
Common peptide precursor, **1**	D-Phe	Gln	Trp	Ala	Val	Gly	His	Leu-HMBA-Resin
D-Phe^6-BBN [6–13] ethyl amide, **2**	D-Phe	Gln	Trp	Ala	Val	Gly	His	Leu-$NHCH_2CH_3$
D-Phe^6-BBN [6–13] butyl amide, **3**	D-Phe	Gln	Trp	Ala	Val	Gly	His	Leu-$NH(CH_2)_3CH_3$
D-Phe^6-BBN [6–13] methyl ester, **4**	D-Phe	Gln	Trp	Ala	Val	Gly	His	Leu-OCH_3
D-Phe^6-BBN [6–13] propyl ester, **5**	D-Phe	Gln	Trp	Ala	Val	Gly	His	Leu-$O(CH_2)_2CH_3$
D-Phe^6-BBN [6–13] Hydrazide, **6**	D-Phe	Gln	Trp	Ala	Val	Gly	His	Leu-$NHNH_2$

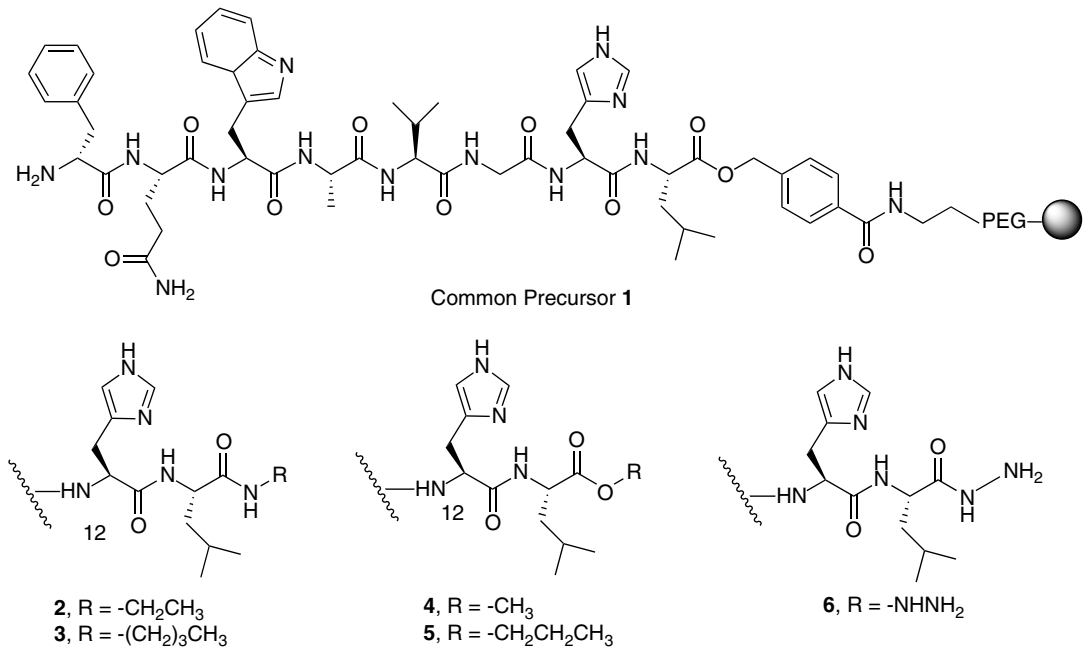

Figure 1 Structures of common peptide precursor **1** and synthesized peptides **2–6**.

A manual SPPS reaction vessel with a sintered glass funnel fitted with a three-way stopcock was obtained from Chemglass, Inc., Vineland, NJ. Agitation of the resin beads is achieved by bubbling N_2 from the bottom of the reaction vessel.

HPLC analysis and separations were performed on a Beckman Coulter Gold Chromatography system coupled to a 168-photodiode array UV detector, a 507e autosampler and equipped with 32 Karat software package (Beckman Coulter, Fullerton, CA). Analyses were performed with a C-18 Kromosil column (5 μm, 100 Å, 150 mm × 4.5 mm), Keystone scientific, Inc. (San Jose, CA). HPLC solvents consisted of 0.1% aqueous TFA, (solvent A) and MeCN containing 0.1% TFA (solvent B) at a 1.0 ml/min flow rate and UV detection at 214 and 280 nm.

A Waters (Milford, MA) NovaPak C18 column (3.9 × 300 mm) was used for the HPLC analyses coupled to a Finnigan TSQ700 mass spectrometer (Thermo Finnigan, San Jose, CA). A Centrific centrifuge and a Sonicator model FS60, Fisher Scientific, Fair Lawn, NJ, USA were used as needed. Peptide resin solutions were dried using a Speed Vac Concentrator model SC110, Savant Instrument Inc., Holbrook, NY, USA.

Yields of **2**–**6** are based on the substitution level of leucine to the resin. HPLC analysis accomplished with the following methods: Step 1: Sequential linear from 10 to 50% solvent B in 30 min, step 2: 50–80% solvent B in 5 min, step 3: remain steady at 80% solvent B for 5 min, step 4: from 80 to 10% solvent B in 2 min, and step 5: remain steady at 10% solvent B for 10 min. The products were stored at 4 °C.

General Procedures for Qualitative Colorimetric Tests Used

2-Methyl red – diphenyldichlorosilane test for free –OH groups. A few pre-swelled HMBA resin beads were washed with THF several times and dried. 200 μl of a 10% solution of TEA in DCM was added to 5 mg dry resin beads followed by 100 μl diphenyldichlorosilane. After 10 min at room temperature (rt), the resin beads are filtered and washed twice with 10% TEA in DCM. The resin beads are suspended in 300 μl DMF containing 0.75% (w/v) of methyl red. The resin beads washed with fresh DMF and DCM until the filtrate became colorless. If the resin beads remain orange/red-colored at this time, it is a positive indication for the presence of free –OH groups.

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TNBSA colorimetric test for free –NH_2 groups. A sample of peptidyl resin beads (~10 mg) is washed with THF twice for 2 min. A few drops of 10% DIPEA in NMP are added followed by two drops of TNBSA. If the resin does not show any red color in a few minutes, the test outcome is negative, and the coupling is considered complete.

Synthesis of Common Peptide Precursor 1

In situ formation of fmoc-leucine-anhydride. Fmoc-Leu-OH (1500 μmol, 530.1 mg), 10 equivalents relative to the resin loading, was dissolved in 4.5 ml DCM. Two drops DMF were added to help dissolve the amino acid. DIPCI (750 μmol, 94.5 mg) in dry DCM (116 μl), was added and the mixture was stirred for 20 min at 0 °C under a $CaCl_2$ drying tube. A few drops of dry DMF were added to dissolve the gelatinous precipitate. DCM was removed by evaporation and the anhydride was used without characterization.

Coupling of Fmoc-Leu-anhydride to HMBA resin. HMBA resin (150 μmol, 652.17 mg), with substitution level of 0.23 μmol/mg, was swelled with DMF (~3 times bed volume). The resin was washed 10–20 times with DMF, each time for 2 min. A small sample of the resin was tested with methyl red and a positive test was obtained. Fmoc-Leu anhydride (5 equivalents) was added to the resin along with DMAP (0.1 equivalent, 15 μmol, 1.83 mg) in DMF (50 mM). The mixture stood for 1 h at rt under N_2 agitation and the peptide-resin was washed with DMF twice for 2 min. A negative methyl red test was obtained.

Assessment of Coupling Efficiency

A small sample of the resin beads was washed several times with THF. The resin was dried and weighed along with 10 μmol of Fmoc-Gly-OH (2.97 mg), Fmoc-XAL-resin (66.67 mg) and Fmoc-Rink amide resin (16.39 mg). The Fmoc group of each sample was removed with ~1 ml of 25% piperidine/DMF for 60–75 min followed by filtration. An aliquot (140 μl) of each solution was transferred to an autosampler vial and diluted with 1.26 ml NMP. Aliquots (20 μl) of each solution were analyzed for the piperidine-dibenzylfulvene adduct by HPLC, using the following gradient conditions: Step 1: from 20 to 60% solvent B in 20 min, step 2: from 60 to 80% solvent B in 2 min, step 3: remain steady at 80% solvent B for 10 min, step 4: from 80 to 20% solvent B in 2 min, step 5; remain steady at 20% solvent B for 10 min. Comparison of peak areas of the adduct released from each sample yielded an accurate measurement of the Fmoc-Leu attached to the HMBA resin. A coupling efficiency of 80–85% was generally observed, confirming the qualitative results of the methyl red colorimetric test. *Note:* If the percentage of the first amino acid attachment is less than 75%, the coupling of Fmoc-Leu-anhydride should be repeated.

Excess (~4 ml) 5% acetic anhydride and 6% DIPEA in NMP was added to the peptidyl resin at rt with gentle N_2 agitation for 10–15 min. The resin was washed with NMP several times. The absence of any un-reacted OH-groups was verified by using the methyl red test and the TNBSA test was negative.

Deprotection of Fmoc-Leu-HMBA resin. About 4–5 ml of 25% v/v piperidine in DMF was added to the Fmoc-Leu-HMBA resin and after 25 min under gentle N_2 agitation at rt, the reagents were drained and the resin was washed twice with DMF. This cycle was repeated and then the resin was washed with NMP twice. The TNBSA test was positive.

In situ carboxy activation of Fmoc-His(Trt)-OH. Three equivalents, relative to the resin loading, of Fmoc-His(Trt)-OH (450 μmol, 278.86 mg) was dissolved in 9 ml NMP. HBTU (405 μmol, 153.62 mg) and HOBt (450 μmol, 68.89 mg) were dissolved in 3 ml DMF. These two solutions were mixed and DIPEA (900 μmol, 116.33 mg) was added and vortexed thoroughly for 10–15 min. This solution was added directly to Leu-HMBA resin and agitated gently for 30 min under N_2 at rt. The reagents were drained and washed twice with NMP and the cycle was repeated. The TNBSA test was negative.

Peptide chain elongation. The reaction cycle for introduction of each new amino acid (Fmoc-Gly-OH, Fmoc-Val-OH, Fmoc-Ala-OH, Fmoc-Trp(Boc)-OH, Fmoc-Gln(Trt)-OH, Fmoc-D-Phe-OH) is Fmoc deprotection followed by coupling of the now free amino group with the activated acid of the incoming amino acid as described above. After the last amino acid elongation, the *N*-terminal Fmoc group is removed.

TFA side-chain deprotection to yield 1. The acid sensitive side-chain protecting groups for histidine, tryptophan and glutamine residues were removed using a deprotection-scavenger cocktail that consists of 95 TFA : 2.5 H_2O : 2.5 TIS. The dry peptidyl resin was mixed with the cocktail (10–20 ml cocktail/gm peptidyl resin) for 1 h at rt. The cocktail was filtered and the resin was washed twice with TFA and then 5 times with NMP to yield **1. 1** was re-suspended in the minimum possible amount of NMP and used in the following reactions.

D-Phe^6-BBN (6–13)-ethylamide, 2. A sample of **1**, resuspended in a minimum amount of NMP, was placed in a dry round bottom flask and cooled in a dry ice/acetone bath. Gaseous ethylamine was condensed over 10 min into the flask, and the inlet was then closed. After 18 h at rt, the contents of the flask were transferred to a SPPS reaction vessel and washed three times with MeCN each time for 5 min. The filtrate was evaporated and **2** was precipitated by addition of cold diethyl ether and centrifuged for 10 min at a speed of 7.5 *g*. The diethyl ether was decanted and the peptide was washed 3 more times with fresh diethyl ether. **2** was dried to yield 17 mg (16%) as a white powder; $k' = 6.11$, 93% purity. ESI-MS (m/z): Theory: $C_{49}H_{69}O_9N_{13} = 984.15$; actual: $(M + H)^+ = 984.4$ and $(M + 2H)^{2+}/2 = 493.1$.

D-Phe^6-BBN (6–13)-butylamide, 3. A sample of **1** was swelled with THF (12 ml/g) for 1 h. A cleavage solution of TEA/Butylamine/THF (1 : 5:5), 50 ml/g peptidyl resin was added and the reaction was stirred overnight (~18 h) at 50 °C. The solution was filtered and the resin was washed first with butylamine/THF and then with DCM for three times each time for 2 min. The combined washes were reduced to ~1 ml of a yellow oil and **3** was precipitated by addition of cold diethyl ether and treated as for **2** to yield 15 mg (13%) as a white powder; $k' = 7.43$, 91.5% purity. ESI-MS (m/z): Theory: $C_{51}H_{73}O_9N_{13} = 1012.21$; actual: $(M + H)^+ = 1012.6$ and $(M + 2H)^{2+}/2 = 507.43$.

D-Phe^6-BBN (6–13)-methyl ester, 4. A sample of **1** was placed in a dry round bottom flask with sufficient NMP to make the

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resin mobile. A cleavage solution of DIPEA/Methanol/DMF (1 : 5 : 5 v/v) (~50 ml cleavage mixture/1 g peptide resin) was then added. After 1 h at rt, 3 h at 50 °C using sonication, and overnight at 50 °C, the solution of methanol/DMF was filtered. The resin was washed with methanol/DMF three times for 2 min and then with TFA into a separate tube to remove any methanol-insoluble peptide. The combined DMF/methanol solutions and TFA solution were reduced to ~1 ml yellow oil. **4** was precipitated by addition of cold diethyl ether and treated as for **2** to yield 34 mg (32%) as a white fluff; $k' = 6.61$, 94% purity. ESI-MS (m/z): Theory: $C_{48}H_{66}O_{11}N_{12} = 971.11$; actual: $(M + H)^+ = 971.47$ and $(M + 2H)^{2+}/2 = 486.47$.

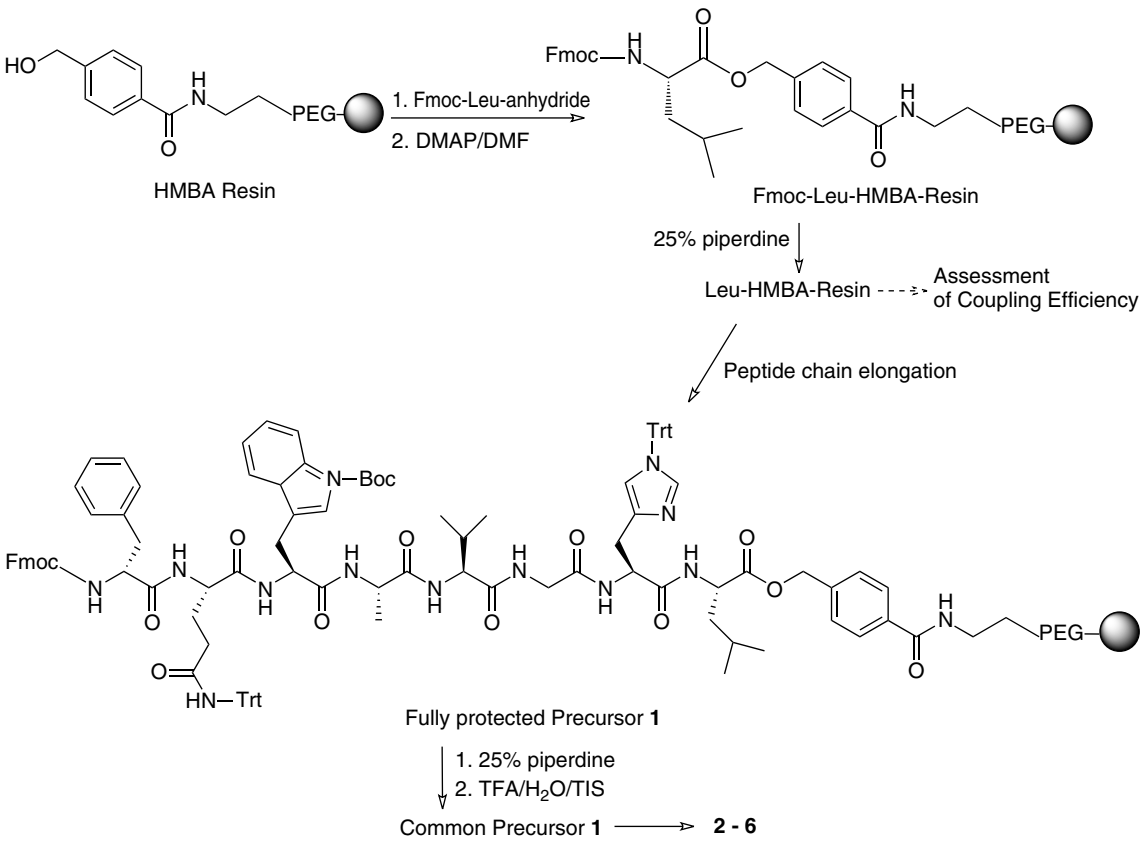
D-Phe*^{6}*-BBN (6–13)-propyl ester, 5. A sample of **1** was swelled with THF (12 ml/g) for 1 h. A cleavage solution of TEA/propanol/THF (1 : 5 : 5), 50 ml/g peptidyl resin and one–two small crystals of KCN (catalyst) were added and the reaction was stirred overnight (~18 h) at 50 °C. The mixture was filtered and the resin was washed first with propanol/THF and then with DCM three times for 2 min. The filtrate was reduced to ~1 ml yellow oil. **5** was precipitated by addition of cold diethyl ether and treated as for **2** to yield 28 mg (25%) as a white fluff; $k' = 8.07$, 97% purity. ESI-MS (m/z): Theory: $C_{50}H_{70}O_{10}N_{12} = 999.17$; actual: $(M + H)^+ = 999.53$ and $(M + 2H)^{2+}/2 = 499.77$.

D-Phe*^{6}*-BBN (6–13)-hydrazide, 6. A sample of **1** was suspended in a minimum amount of DMF to make the resin mobile to N_2 agitation. 5% v/v hydrazine hydrate in DMF (20 ml/gm) was added and after ~18 h at rt, the solution was filtered. The resin was washed three times for 2 min with a resin bed volume of DMF. The resin was subjected to a wash with TFA into a separate flask to remove any DMF insoluble peptide. The combined washes were reduced to 1 ml yellow oil, and **6** was precipitated by addition of cold diethyl ether and treated as for **2** to yield 25 mg (23%) as white granules; $k' = 10.11$, 91% purity. ESI-MS (m/z): Theory: $C_{47}H_{66}O_9N_{14} = 971.12$; actual: $(M + H)^+ = 971.59$ and $(M + 2H)^{2+}/2 = 486.56$.

RESULTS AND DISCUSSION

Five *C*-terminal modified peptides **2–6** were prepared from a common precursor (**1**) synthesized on solid phase using HMBA resin by the nucleophilic cleavage strategy shown in Scheme 1. The swelling properties of HMBA resin required preparation of the peptides via a manual peptide reactor rather than an automatic peptide synthesizer.

The attachment of the first amino acid residue (leucine) was accomplished by coupling the symmetric Fmoc-leucine-anhydride rather than the activated Fmoc-leucine to the hydroxyl functionalized HMBA resin. The formation of the anhydride requires absolutely dry environment, reagents and glassware. A coupling efficiency of 81–85% was generally achieved in this reaction. If, for any reason, the coupling efficiency is found to be lower than 75%, it is recommended that the coupling step should be repeated. The systematic



Scheme 1

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construction of the common precursor **1** was achieved in a manual reaction vessel with mixing the contents by nitrogen agitation and removing the solvents by applying vacuum. The standard cycle of Fmoc deprotection of the *N*-terminus amino acid residue, followed by activation of the incoming amino acid was used for each elongation step. The transient protecting groups chosen for the amino acid side chains were the Boc group for tryptophan and the trityl group for glutamine and histidine. The *in situ* activations of the Fmoc-protected amino acids (3 equivalents to 1 equivalent of resin) were carried out using HBTU (2.7 equivalents) and HOBt (3.0 equivalents) in the presence of DIPEA (6 equivalents) as the tertiary base. Two coupling cycles were performed in order to minimize the production of deletion mutants. A TNBSA test was performed at every coupling step to check the completeness of the reaction.

With the peptide precursor **1** in hand, protocols were designed to test cleavage of the base sensitive peptide ester linkage bond of the HMBA resin. Unlike the well-known and quite well established TFA cleavage protocol, procedures for nucleophilic cleavage are not as well described. Our initial trials were based upon general recipes described in catalogs or reference texts [2–6,21]. In our hands, these protocols yielded either negative or ambiguous results rather than the desired peptides. Several attempts were made in our labs using the nucleophile in the presence of DIPEA in DMF (1 : 5:5 v/v) ~50 ml/gm for 1 h at rt and 3 h at 50 °C using sonication. None of these reaction conditions provided material that LC/ESI-MS could verify as the desired peptide product (data not shown). In addition, utilizing an appropriate solvent with a lower boiling point would obviate the need for DMF, a solvent that is sometimes problematic to remove entirely; therefore, we investigated the use of THF.

Additional cleavage procedures using the alcohol or the requisite amine with TEA in THF (1 : 5 : 5 v/v) ~50 ml/gm at different reaction conditions included 1 h at rt, 3 h at 50 °C using sonication, and overnight at 50 °C. Of these conditions, we achieved marked success with the last procedure. The crude bombesin analogs were characterized using analytical HPLC and LC/ESI-MS. The peptides were obtained in yields from 13 to 32%, calculated as the percentage of micro moles final product obtained compared to the initial micro moles Leu-HMBA-resin utilized. These final yields reflect not only the efficiency of the cleavage reaction but also the yields inherent in the peptide elongation steps. Thus, these final yields are conservative estimates for the cleavage step alone. Generally, the synthesis of *C*-terminal modified peptides is sensitive to steric effects which may contribute to a lower cleavage yield. The purity of these peptides was extremely high (91–97%). Extending the cleavage time did not increase the yield by any measurable amount. To avoid any undesirable effect on the heat-sensitive peptide bond, we did not examine the effect of a higher temperature for the cleavage reactions.

In the above reactions, cleavage conditions were conducted on the fully deprotected peptide precursor **1**. The reverse protocol, i.e. removing the acid-labile side-chain protecting groups after the nucleophilic cleavage, was also studied in a preliminary fashion. We were able to obtain our peptides in these cases, but in lower absolute purity (data not shown). Since high purity of the peptide products is a requisite for subsequent biological evaluation, we focused our efforts on optimizing the side-chain deprotection/cleavage reaction sequence.

The development of methodology to achieve the syntheses of *C*-terminal modified functionalities using the nucleophilic cleavage approach provides several advantages. One of the major advantages of using the nucleophilic cleavage strategy is the ability to prepare a series of peptides with a wide variety of *C*-terminal functionalities from preparation of a single precursor. This advantage not only reduces the number of synthetic steps but also minimizes the purchase of specialized expensive resins for the preparation of specific *C*-terminal moieties. Furthermore, base-sensitive cleavage of the ester bond before or after deprotection of acid-labile side-chain protecting groups provides additional flexibility in synthesis.

On the other hand, the formation of the initial ester linkage is more difficult than that of an amide bond. Achieving a high substitution level of the first amino acid onto the resin is more challenging. Depending upon the choice of amino acid, the possibility of epimerization and dipeptide formation exists. Therefore, precautions to maximize yield include the use of extremely dry glassware and reagents and the use of the minimum possible solvent volume during the coupling steps to maximize the reagent concentration.

In this study, sufficiently strong, but sterically unhindered, nucleophiles were utilized. These procedures should be practical regardless of the peptide construct chosen. The peptide constructs described here do not contain the internal nucleophilic side chains found with lysine or serine residues; however, the concentration of external nucleophile used in the cleavage reaction is in excess. Therefore, it is unlikely that either side chain would compete successfully in the cleavage reaction to form oligomers. If this undesirable side reaction were shown to occur, the order of steps would be modified to cleave the peptide prior to the side chain deprotection.

Less positive outcomes would be possible if cleavage were attempted with weaker or sterically hindered nucleophiles, or whether the cleavage reaction was attempted on sterically hindered esters. In this case, using harsher cleavage conditions such as extended cleavage time, elevated temperature or repeating the cleavage process using fresh reagents may become necessary for achieving successful cleavage. Reactions

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should be modeled in these cases prior to large-scale production.

CONCLUSION

In conclusion, synthetic details of the solid phase synthesis of five bombesin analogs with different functional groups at the *C*-terminus are reported. The crude peptides are obtained in very high purity by the methods described. The optimized synthetic strategy is very convenient for preparation of these bombesin analogs that possess different moieties in the *C*-terminus.

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