A Convenient Approach to Synthesizing Peptide C-Terminal *N*-Alkyl Amides

Wei-Jie Fang,* Tatyana Yakovleva, Jane V. Aldrich
Department of Medicinal Chemistry, The University of Kansas, Lawrence, KS 66045

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ABSTRACT:

Peptide C-terminal N*-alkyl amides have gained more attention over the past decade due to their biological properties, including improved pharmacokinetic and pharmacodynamic profiles. However, the synthesis of this type of peptide on solid phase by current available methods can be challenging. Here we report a convenient method to synthesize peptide C-terminal* N*-alkyl amides using the well-known Fukuyama* N*-alkylation reaction on a standard resin commonly used for the synthesis of peptide C-terminal primary amides, the peptide amide linker-polyethylene glycol-polystyrene (PAL-PEG-PS) resin. The alkylation and* o*NBS deprotection were conducted under basic conditions and were therefore compatible with this acid labile resin. The alkylation reaction was very efficient on this resin with a number of different alkyl iodides or bromides, and the synthesis of model enkephalin* N*-alkyl amide analogs using this method gave consistently high yields and purities, demonstrating the applicability of this methodology. The synthesis of* N*-alkyl amides was more difficult on a Rink amide resin, especially the coupling of the first amino acid to the* N*-alkyl amine, resulting in lower yields for loading the first amino acid onto the resin. This method can be widely applied in the synthesis of peptide* N*-alkyl amides. © 2011 Wiley Periodicals, Inc. Biopolymers (Pept Sci) 96: 715-722, 2011.*

Keywords: peptide C-terminal N*-alkyl amides; Fukuyama amine synthesis*

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INTRODUCTION

Solid-phase peptide synthesis (SPPS), first developed by R. Bruce Merrifield,[1] permits the rapid synthesis of peptides and related biologically active compounds. Because SPPS almost always starts from the C-terminus of the peptide, C-terminal modifications of peptides are usually more difficult to introduce than modifications at the N-terminus.

C-Terminal amide alkylation of peptides can have significant effects on their biological properties. Groups such as a C-terminal ethyl amide can increase stability toward peptidases,[2,3] and can also increase affinity for specific biological targets.[4–6] In the case of lutenizing hormone-releasing hormone (LH-RH), several *N*-ethyl and *N*-methyl amide derivatives of LH-RH analogs are four to five times more potent in releasing pituitary luteinizing hormone than the corresponding primary amide peptides.[4,5] In addition, C-terminal alkylation can increase lipophilicity and reduce hydrogen bonding potential, which can facilitate the penetration of peptides across biological membranes and/or improve pharmacokinetic properties.[7,8] Therefore, C-terminal amide alkylation has considerable potential for application to therapeutically relevant peptides.

Given the interest in these modified peptides, a number of approaches to the synthesis of peptide C-terminal *N*-alkyl amides have been described.[9] However, the synthesis of this

Correspondence to: Jane Aldrich; e-mail: jaldrich@ku.edu
**Present affiliation:* Department of Chemical and Biological Engineering, University of Colorado, Boulder, CO 80309
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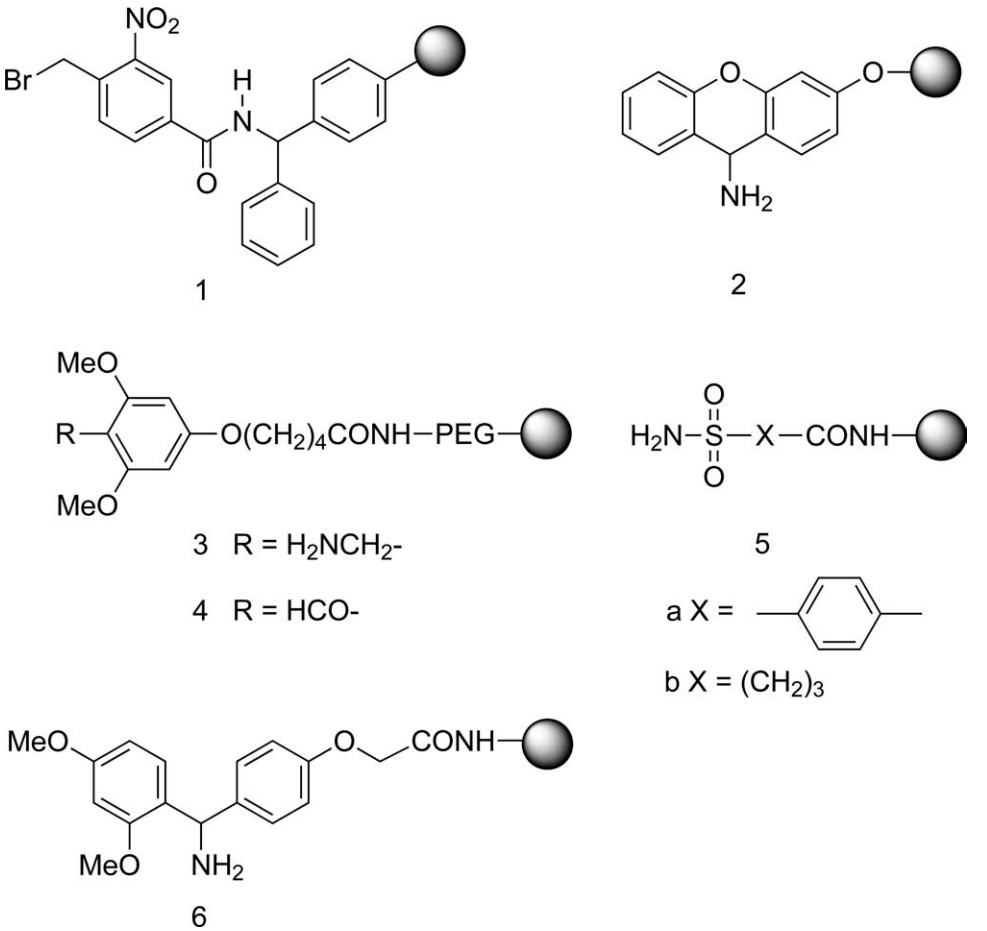
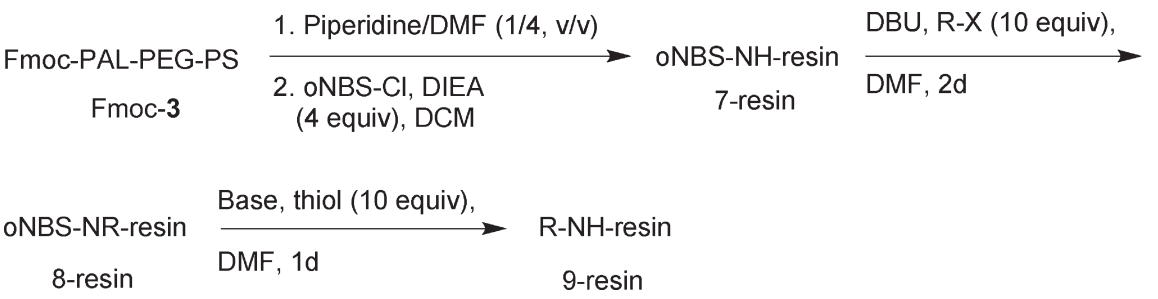


FIGURE 1 Structures of resins **1–6.**

type of modified peptide on solid phase is not straightforward. One approach used is to first synthesize the corresponding peptides with a C-terminal carboxylic acid. Following cleavage from the resin the free carboxylic acid is then activated and coupled to a primary or secondary amine in solution.[10] This approach has some limitations, however, as other carboxylic acid groups, i.e. those on the side chains of Asp and Glu, can also react, and racemization may occur at the C-terminal amino acid.[11] A related approach is aminolysis of a resin-bound ester. For example, a 4-bromomethyl-3-nitrobenzamidobenzyl polystyrene (4-bromomethyl-Nbb) resin (**1**, Figure 1), developed by Nicolas et al., has been used for the solid phase synthesis of peptide C-terminal *N*-alkyl amides using the Boc (*tert*-butyloxycarbonyl) synthetic strategy.[12] This approach is not compatible with the standard Fmoc (9-fluorenylmethoxycarbonyl) synthetic strategy, however, since the secondary amine piperidine used for removal of the Fmoc group following each coupling can also aminolyze the resin-bound peptide ester.[12] Similarly peptide C-terminal *N*-alkyl amides have been prepared by aminolysis of peptides synthesized on an oxime resin,[13] but again this resin is only compatible with the Boc synthetic strategy.

Another approach involves the use of a modified resin linker for introduction of an alkyl amine onto the resin before peptide chain assembly begins. For example, reductive amination of the 9-aminoxanthen-3-yloxymethylpoly(styrene) resin (**2**, Figure 1) with an aldehyde followed by standard peptide synthesis afforded C-terminal alkylated amides.[14] However, reductive alkylation on solid phase with an excess of aldehyde often results in dialkylation[15]; this essentially caps the resin, since an amino acid cannot couple to a tertiary amine, decreasing the yield of the desired peptides. It was also reported that the PAL-PEG-PS (Peptide Amide Linker-polyethylene glycol-polystyrene) resin (**3**, Figure 1), a standard resin for the preparation of peptide primary amides, was not applicable for the synthesis of peptide C-terminal alkylated amides by this methodology because the imine intermediate could not be obtained.[14] Alternatively, reductive amination of an aldehyde-containing resin such as one containing the backbone amide linker[16,17] (BAL, **4**, Figure 1)



SCHEME 1 Introduction of the C-terminal *N*-alkyl groups onto the PAL-PEG-PS-resin.

followed by peptide chain assembly can be used to prepare peptides with a C-terminal *N*-alkyl amide. This approach, however, has been reported only for higher molecular weight amines,[18–21] and could be difficult with smaller, highly volatile amines such as ethylamine.

Another commercially available resin used for the synthesis of these C-terminally modified peptide amides is the "safety catch" resin (**5**, Figure 1).[3,6,22–25] With this resin peptide assembly is first completed, and then the resin is activated by treatment with a reagent such as iodoacetonitrile to yield the *N*-alkyl-*N*-acylsulfonamide; the peptide is then cleaved from the resin with nucleophiles such as amines to yield the *N*-alkyl amide.

Efficient SPPS methodologies for the preparation of *N*-alkyl amides would facilitate the preparation of this important type of peptide. Here we report a convenient method for synthesizing peptide C-terminal *N*-alkyl amides using the standard PAL-PEG-PS resin (**3**) and comparison to the synthesis on a resin containing the Rink amide linker (**6**). We applied a well-known *N*-alkylation reaction, the Fukuyama amine synthesis,[26,27] to the preparation of peptide C-terminal *N*-alkyl amides. This type of reaction has been applied to the synthesis of *N*-methylamino acid-containing peptides by both solid and solution phase methods.[28–31] However, to our knowledge this type of reaction has not been reported previously for the synthesis of peptide C-terminal *N*-alkyl amides.

RESULTS AND DISCUSSION

Introduction of the C-Terminal *N*-Alkyl Group

The synthesis of the peptide C-terminal *N*-alkyl amides was examined on the Fmoc-PAL-PEG-PS resin (Fmoc-**3**, 0.19 mmol/g, Scheme 1). The Fmoc group was first removed using piperidine in *N*,*N*-dimethylformamide (DMF, 1/4, v/v) to give the free amine, which was then reacted with 4 equiv each of *ortho*-nitrobenzenesulfonyl chloride (*o*NBS-Cl) and *N*,*N*-diisopropylethylamine (DIEA) in dichloromethane (DCM). The completion of this reaction was monitored by the ninhydrin test.[32] Cleavage of an aliquot of the resin afforded pure *o*NBS-NH_2 as confirmed by HPLC (5%–50% MeCN with 0.1% trifluoroacetic acid (TFA) over 45 min at 1 mL/min, $t_R = 3.8$ min, see below).

The newly formed sulfonamide (**7**-resin) was deprotonated using 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in DMF[29] and the resulting anion then reacted with several different alkyl halides (Scheme 1). The yields of these reactions were monitored by HPLC analysis of aliquots for the appearance of *o*NBS-NHR (**8**) and the disappearance of *o*NBS-NH_2 (**7**). The results for these reactions are shown in Table I. The conversion was quantitative for most of the alkylating reagents (methyl iodide, ethyl iodide, allyl bromide, propargyl bromide, cyclopropylmethyl (CPM) bromide, and benzyl bromide) after reaction at room temperature for 2 days (Table I, entries A–F), except for phenylethyl chloride and phenylethyl bromide (Table I, entries G–K). Even with potassium iodide (KI) as a catalyst and/or heating the reaction to 80°C (Table I, entries H and I), there was no apparent conversion when the alkylating reagent was the less reactive phenylethyl chloride. With phenylethyl bromide there was only 20% conversion after reaction for 2 days at room temperature (Table I, entry J). Increasing the reaction temperature to 80°C did not increase the yield (21%, Table I, entry K) under the same reaction conditions.

We next examined various conditions for *o*NBS deprotection[26] from the *o*NBS-N(Et)-resin using different bases and thiols (Table II). The completion of this reaction was monitored by the disappearance of the *o*NBS-NH-Et by HPLC. The use of 4-dimethylaminopyridine (DMAP) resulted in only partial cleavage of the *o*NBS group (Table II, entry A). Use of the stronger base DBU resulted in complete removal of the *o*NBS, likely as a result of its stronger basicity compared to DMAP and more efficient deprotonation of the thiol group. Different thiols (2-mercaptoethanol, thiophenol, and 2,2′-(ethylenedioxy)diethanethiol ($(HSCH_2CH_2OCH_2)_2$)) were also investigated; they all resulted in complete deprotection of the *N*-ethylamide under these reaction conditions (Table II, entries B–D). The nonvolatile 2,2′-(ethylenedioxy)-diethanethiol was subsequently used in combination with

Table I Yields of the Alkylation Reactions With Different Alkyl Halides on the PAL-PEG-PS Resin, and the Retention Times (t_R) of the Resulting *o*NBS-NH-R[a]

Entry	R-X	Yield (%)/t_R (min) of *o*NBS-NH-R[b,c]	Entry	R-X	Yield (%)/t_R (min) of *o*NBS-NH-R[b,c]
A	Me-I	>98/7.9	G	$PhCH_2CH_2Cl$	<5
B	Et-I	>98/12.7	H	$PhCH_2CH_2Cl$/KI	<5
C	All-Br	>98/14.9	I	$PhCH_2CH_2Cl$/KI[d]	<5
D	Propargyl-Br	>98/12.9	J	$PhCH_2CH_2Br$	20/31.4
E	CPM-Br	>98/20.1	K	$PhCH_2CH_2Br$[d]	21/31.6
F	Bn-Br	>98/27.0			

[a] After reaction for 2 days at room temperature except where noted.
[b] The yields were determined from HPLC chromatograms by comparing the relative area under the peaks of *o*NBS-NH-R (**8**) with nonalkylated *o*NBS-NH_2 (**7**).
[c] HPLC conditions: 5–50% solvent B over 45 min (solvent A = H_2O, solvent B = MeCN, both containing 0.1% TFA) at 1 mL/min, monitored at 214 nm.
[d] At 80°C.

DBU to deprotect the *o*NBS from the other alkylated resins. To the best of our knowledge the use of 2,2′-(ethylenedioxy)-diethanethiol to deprotect the *o*NBS group has not been previously reported.

While *o*NBS was completely removed from most of the *N*-alkyl resins using DBU and 2,2′-(ethylenedioxy)diethanethiol as the base and thiol, respectively (Table III, entries A–E), the deprotection of the *o*NBS-N(CPM)-resin turned out to be difficult (Table III, entry F). Only a trace amount of the *o*NBS was removed under standard conditions, and increasing the temperature to 80°C only increased the deprotection to around 30% (entry G). This sluggish reaction is likely due to steric hindrance by the CPM group. Thiophenol was found to be a much better reagent to remove the *o*NBS group from this *N*-alkylated resin (Table III, entry H), probably due to increased nucleophilicity. Any remaining unreacted *o*NBS group would act as a capping group and would not interfere with subsequent peptide synthesis.

We also investigated the use of this methodology on a Rink amide resin to prepare peptide C-terminal alkyl amides using the reaction conditions developed for the PAL-PEG-PS resin. *o*NBS-Cl was reacted with the amine on a Rink-PEG-PS resin as described above; completion of the reaction was monitored with ninhydrin. The alkylation of the sulfonamide was then examined with ethyl iodide and also the bulky alkyl halide CPM bromide; the latter was examined to determine whether steric hinderance from the Rink amide linker would affect more difficult alkylations. The alkylation with ethyl iodide proceeded almost to completion, yielding 95% of the *o*NBS-NH-R after reaction at room temperature for a total of 3 days (see Experimental). The alkylation with CPM bromide, however, was only 76% complete using these conditions, as determined by HPLC of an aliquot, consistent with a slower reaction due to steric hinderance from the Rink amide linker. The conversion to *o*NBS-N(CPM)-resin was increased to 93% by an additional treatment with CPM bromide at 80°C for one day. The *o*NBS group was then removed as described above; disappearance of the *o*NBS-NH-R peak in the HPLC of aliquots verified that the reaction on both resins was complete.

Table II Exploration of Different Conditions for the Cleavage of *o*NBS From the *o*NBS-N(Et)-PAL-PEG-PS Resin (Base/Thiol/DMF = 1/1/2, v/v/v, 1 Day at Room Temperature)

Entry	Base	Thiol	Yield (%)[a]
A	DMAP	$HSCH_2CH_2OH$	24
B	DBU	$HSCH_2CH_2OH$	>99
C	DBU	PhSH	>99
D	DBU	$(HSCH_2CH_2OCH_2)_2$	>99

[a] Determined by HPLC; see Table I for conditions.

Table III Cleavage of the *o*NBS Group From Different *o*NBS-N(R)-PAL-PEG-PS Resins (DBU/2,2′-(ethylenedioxy)diethanethiol/DMF = 1/1/2, 1 Day at Room Temperature)

Entry	R	Yield (%)[a]	Entry	R	Yield (%)[a]
A	Me	Quantitative	E	Bz	Quantitative
B	Et	Quantitative	F	CPM	Trace
C	Allyl	Quantitative	G	CPM[b]	30%
D	Propargyl	Quantitative	H	CPM[c]	Quantitative

[a] Determined by HPLC; see Table I for conditions.
[b] At 80°C.
[c] Thiophenol used instead of 2,2′-(ethylenedioxy)diethanethiol.

Table IV Resin Loadings of Different Fmoc-Leu-N(R)-PAL-PEG-PS Resins, as Determined by Quantitative Fmoc Analysis

Entry	Resin	Fmoc Quantititation (mmol/g)[a]	Loading Yield (%)
A	Fmoc-Leu-N(Me)-resin	0.171 ± 0.001	92
B	Fmoc-Leu-N(Et)-resin	0.169 ± 0.004	91
C	Fmoc-Leu-N(All)-resin	0.171 ± 0.001	92
D	Fmoc-Leu-N(Propargyl)-resin	0.176 ± 0.003	95
E	Fmoc-Leu-N(CPM)-resin	0.175 ± 0.001	95
F	Fmoc-Leu-N(Bz)-resin	0.160 ± 0.001	87

[a] Mean ± SEM of three samples.

Peptide Synthesis and Analysis

To evaluate this method of synthesizing peptide C-terminal *N*-alkyl amides we then introduced Fmoc-Leu-OH onto the *N*-alkyl amines in preparation for the synthesis of the C-terminal *N*-alkyl amide derivatives of the model pentapeptide Leu-enkephalin, Tyr-Gly-Gly-Phe-Leu-NH-R. Coupling of Fmoc-Leu-OH (using benzotriazole-1-yloxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyBOP), 1-hydroxybenzotriazole (HOBt), and DIEA in DMF (4/4/4/10) as coupling reagents) to the secondary amino groups on the PAL-PEG-PS resin was relatively slow and required double couplings with extended reaction times (overnight). Any unreacted free amino groups were then blocked using an excess of acetic anhydride and DIEA in DMF, and the loading of the first amino acid determined by Fmoc quantitation (Table IV).[11,33] Coupling yields of the first amino acid Fmoc-Leu to these secondary amino groups on the PAL-PEG-PS resin were generally >90% (Table IV).

Coupling of Fmoc-Leu-OH to the *N*-ethyl or *N*-CPM amines on the Rink amide resin, however, proved to be more difficult. Following the double coupling of Fmoc-Leu-OH to the *N*-ethyl resin using the procedure described above and cleavage from the resin with TFA, two products were detected by HPLC that appeared to be Fmoc-Leu-NH-Et (t_R = 36.3 min, 71%) and Fmoc-Leu-NH_2 (t_R = 32.5 min, 29%). The presence of Fmoc-Leu-NH_2 suggests that the amine groups on this resin may not have reacted completely with *o*NBS-Cl, even though a negative ninhydrin test was obtained. An additional coupling of Fmoc-Leu-OH with the more reactive 6-chloro derivative of PyBOP PyClock (6-chlorobenzotriazole-1-yloxy-tris-pyrrolidinophosphonium hexafluorophosphate)[34] increased the yield of Fmoc-Leu-NH-Et to 93%, decreasing the second product to 7% as determined by HPLC. Thus nonalkylated amine represented a small percentage of the resin, and the larger percentage of Fmoc-Leu-NH_2 following the initial double coupling was due to the higher reactivity of the primary amine in the coupling compared to the secondary amine. In the case of the *N*-CPM resin after the initial double coupling Fmoc-Leu-NH-CPM (t_R = 40.3 min) was the minor product by HPLC (42%), and the peak at 32.7 min that appeared to be Fmoc-Leu-NH_2 was the major product (58%). An additional coupling with PyClock increased the yield of Fmoc-Leu-NH-CPM to 86%. Following the coupling with PyClock the resin substitutions for Fmoc-Leu-NH-Et and Fmoc-Leu-NH-CPM were both determined to be 0.11 mmol/g by Fmoc quantitation, corresponding to loading yields of only 48% for the first amino acid on this resin, in contrast to loading yields of >90% for the PAL-PEG-PS resin.

Following the successful installation of the first amino acid, the synthesis of the Leu-enkephalin C-terminal *N*-alkyl amides was continued on the PAL-PEG-PS resin using standard SPPS methods described previously.[35] These peptides were then cleaved from the resin with 95% TFA with 5% water for 2 h. Electrospray ionization mass spectrometry (ESI-MS) analysis showed the desired molecular weights for all of the peptides (Table V), and HPLC analysis indicated

Table V HPLC and MS Analysis of Crude Peptide C-Terminal Alkylated Amides Synthesized on the PAL-PEG-PS Resin

Entry	Peptide Sequence	HPLC[a] t_R (min)/% Purity	ESI-MS (*m/z*, $[M + H]^+$) Calculated	ESI-MS (*m/z*, $[M + H]^+$) Observed
A	Y-G-G-F-L-NH_2	14.6/98.4	555.3	555.3
B	Y-G-G-F-L-NH-Me	14.8/90.4	569.3	569.3
C	Y-G-G-F-L-NH-Et	16.8/97.0	583.3	583.3
D	Y-G-G-F-L-NH-All	17.9/88.0	595.3	595.3
E	Y-G-G-F-L-NH-propargyl	17.6/98.9	593.3	593.3
F	Y-G-G-F-L-NH-CPM	20.0/90.7	609.3	609.3
G	Y-G-G-F-L-NH-Bz	23.5/93.8	645.3	645.3

[a] See Table I for HPLC conditions.

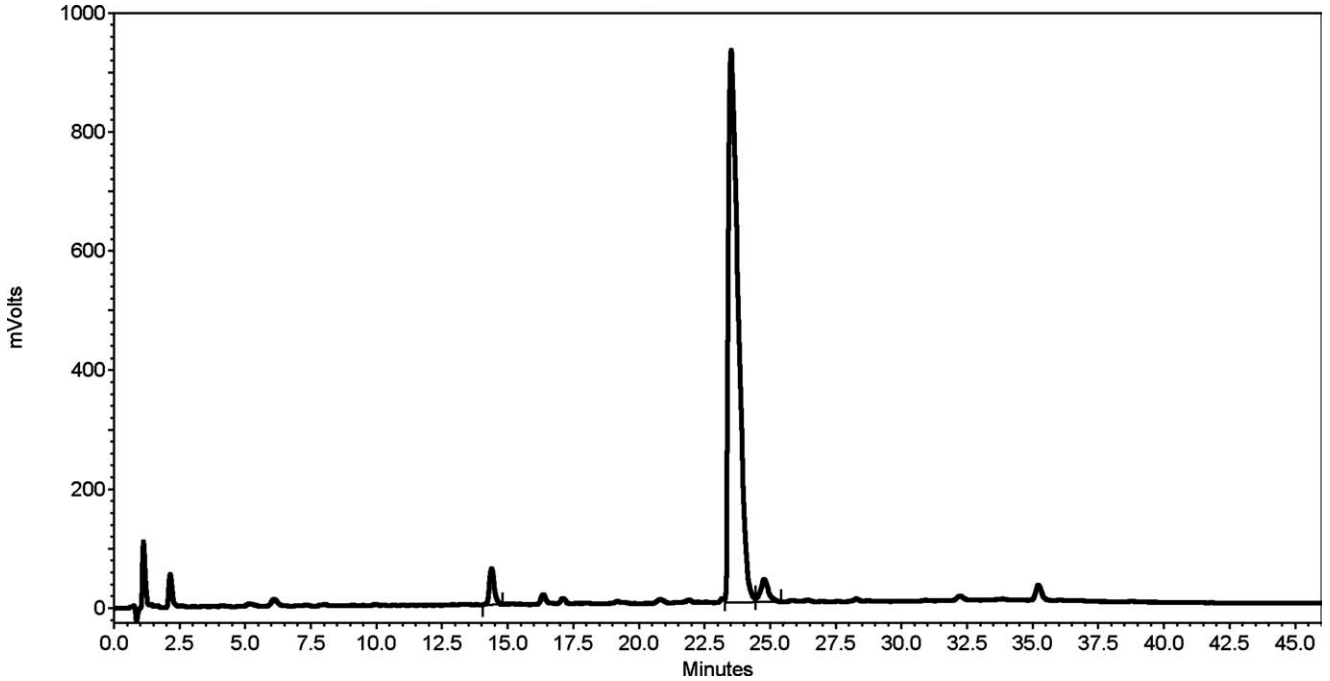


FIGURE 2 HPLC chromatogram of the crude peptide Y-G-G-F-L-NH-Bz synthesized on the PAL-PEG-PS resin. See Table I for HPLC conditions.

that most of these crude peptides had a purity of >90% (Table V). There was no trace of the non-alkylated control peptide Tyr-Gly-Gly-Phe-Leu-NH_2, either in the HPLC or MS spectra (HPLC t_R = 14.6 min, see Table V for conditions; calculated $[M+H]^+$ = 555.3), for any of these peptides. Figure 2 shows the HPLC tracing of crude Leu-enkephalin-NH-Bz (Tyr-Gly-Gly-Phe-Leu-NH-Bz) as an example.

The methodology described here offers an alternative to other methods to synthesize peptide C-terminal *N*-alkyl amides. It is complementary to the use of a BAL resin, especially for the incorporation of small alkyl groups such as methyl or ethyl into the C-terminal amide. As noted earlier, these groups are of considerable interest in the design of therapeutically relevant peptides. In both these methods the C-terminal *N*-alkyl group is installed in the initial steps in the synthesis. Once the C-terminal *N*-alkyl group is introduced, the peptide sequence can be easily varied using standard SPPS methodology, making it especially useful for the synthesis of combinatorial peptide libraries containing a modified C-terminus. In contrast, with the "safety catch" resin the *N*-alkyl group is introduced during the last step when the peptide is cleaved from the resin.[3,6,22–25] There are limitations with regard to the nucleophile used in this reaction, and hindered amines do not always yield the desired peptide.[6]

CONCLUSIONS

In conclusion, we applied a well-known *N*-alkylation reaction to the efficient synthesis of peptide C-terminal *N*-alkyl amides. The acid labile PAL-PEG-PS and Rink amide resins are compatible with the reaction conditions since the alkylation and *o*NBS deprotection reactions are conducted under basic conditions. The alkylation reaction on the PAL-PEG-PS resin proceeded in high yields with a variety of alkyl bromides and iodides at room temperature. We have introduced the use of the nonvolatile 2,2′-(ethylenedioxy)diethanethiol to deprotect the *o*NBS group, which offers a significant advantage over the malodorous thiols typically used in this reaction. The synthesis of the model enkephalin *N*-alkyl amide analogs on the PAL-PEG-PS resin using this methodology gave consistently high yields and purities, demonstrating the applicability of this synthetic strategy. This methodology can be widely applied in the combinatorial synthesis of peptide C-terminal *N*-alkyl amides.

In contrast, the synthesis of *N*-alkyl amides on a Rink amide resin was more difficult than on the PAL-PEG-PS resin. Alkylation of the Rink amide resin with the bulky alkyl halide CPM bromide required modified conditions (elevated temperature) to drive the reaction to close to completion. Coupling to the *N*-alkyl amine on the bulky Rink amide linker was much more difficult than on the PAL-PEG-PS resin, and even an additional third coupling with the more reactive

coupling reagent PyClock resulted in low loading yields for the first amino acid on the Rink amide resin.

EXPERIMENTAL

Materials

All Fmoc-protected amino acids were purchased from Bachem (King of Prussia, PA), Novabiochem (San Diego, CA), Applied Biosystems (Foster City, CA), or Peptides International (Louisville, KY). Fmoc-PAL-PEG-PS resin was purchased from Applied Biosystems, and the Rink amide-PEG-PS resin (NovaSyn TGR resin) was purchased from Novabiochem. PyBOP and PyClock were purchased from Novabiochem. All solvents, methyl iodide, and TFA were purchased from Fisher Scientific (Hampton, NH). HOBt, allyl bromide, benzyl bromide, propagyl bromide, phenylethyl chloride, and thiophenol were purchased from Acros Chemical Co. (Pittsburgh, PA). All other chemicals, including *o*NBS-Cl, DBU, DMAP, ethyl iodide, cyclopropylmethyl bromide, phenylethyl bromide, potassium iodide, 2-mercaptoethanol, 2,2′-(ethylenedioxy)diethanethiol, and piperidine, were purchased from Aldrich Chemical Co. (Milwaukee, WI).

Installation of the C-Terminal *N*-Alkyl Group

The Fmoc group of the Fmoc-PAL-PEG-PS resin (Fmoc-**3**, 0.19 mmol/g) was first removed using piperidine in DMF (1/4, v/v, 2 × 10 min) to yield the free amine, which was then coupled with 4 equiv each of *o*NBS-Cl and DIEA in DCM for 30 min to afford the *o*NBS-NH-resin (**7**-resin, Scheme 1). The completion of this reaction was monitored by the ninhydrin test.[32] Cleavage of an aliquot of the resin afforded pure *o*NBS-NH_2 (**7**, HPLC (5–50% aqueous MeCN containing 0.1% TFA over 45 min at 1 mL/min, monitored at 214 nm): t_R = 3.8 min). The *o*NBS-NH-resin was then treated with an alkyl halide and DBU (10 equiv each) in DMF for 2 days at room temperature to afford the *o*NBS-NR-resins (**8**-resins). The yields of these reactions were monitored by HPLC analysis (see conditions above) of aliquots to monitor the appearance of *o*NBS-NHR and the disappearance of *o*NBS-NH_2 following cleavage from the resin using TFA (Table I). For phenylethyl chloride, KI (0.1 equiv) was also added as a catalyst; the reaction was run at room temperature or at 80°C to increase the conversion rate (Table I).

The *o*NBS-NR-resins (**8**-resins) with quantitative alkylation were then treated with different bases and thiols in DMF (1/1/2) at room temperature for 1 day to afford the RNH-resins (**9**-resins). A small quantity of resin (around 5 mg) was cleaved using TFA (1 mL) for 3 h. Following filtration and washing the resin with TFA (1 mL), the TFA was evaporated and the residue was analyzed by HPLC using the conditions described above. The difference in the absorbance of *o*NBS-NHR before and after *o*NBS deprotection was used to estimate the conversion rate.

The amine of the NovaSyn TGR resin (0.25 mmol/g) was treated with *o*NBS-Cl as described above to yield the *o*-NBS-NH-resin; the reaction was monitored by the ninhydrin test. The resin was then treated with either ethyl iodide or CPM bromide and DBU in DMF, first with 10 equiv each for two days at room temperature as described above, followed by treatment with 20 equiv each for an additional day at room temperature. The yields of these reactions, determined by HPLC as described above, was 95% for *o*NBS-NH-Et and 76% for *o*NBS-NH-CPM. Because of the low yield in the latter case the alkylation with CPM bromide and DBU (20 equiv) was repeated at 80°C for an additional day; this increased the yield of *o*NBS-NH-CPM to 93%.

Coupling of Fmoc-Leu-OH to the *N*-Alkyl Resins

Fmoc-Leu-OH (4 equiv) was double coupled to the **9**-PAL-PEG-PS resins with PyBOP, HOBt, and DIEA (4/4/10) in DMF for 12 h. The reactions were monitored using the chloranil test.[36] Any unreacted free amino groups were capped using an excess of acetic anhydride and DIEA (20 equiv each) in DMF for 30 min. Loading of the first amino acid was then determined by Fmoc quantitation (see below).[11,33]

The *N*-ethyl and *N*-CPM Rink amide resins were initially double coupled with PyBOP, HOBt and DIEA as described above. Aliquots of each resin were cleaved with TFA, and the products analyzed by HPLC using the conditions listed above. The coupling with Fmoc-Leu-OH (4 equiv) was repeated with PyClock,[34] HOAt (1-hydroxyazabenzotriazole) and DIEA (4/4/10) in DMF for an additional 12 h; additional aliquots of each resin were then cleaved with TFA and analyzed by HPLC.

Fmoc Quantitation[11,33]

About 5 mg of resin (three samples for each resin) was deprotected using piperidine (0.4 mL) in DCM (0.4 mL) for 30 min. Methanol (1.6 mL) was then added, and the mixture diluted to a total volume of 10 mL with DCM. The UV absorbance was measured at 301 nm compared to a blank, and the Fmoc loading calculated using an extinction coefficient of 7800 M^{-1} cm^{-1}.

Synthesis of the Model Peptides

Following successful installation of the first amino acid, the synthesis of the Leu-enkephalin C-terminal *N*-alkyl amides was continued on the PAL-PEG-PS resin using the standard SPPS method as described previously.[35] The model peptides were cleaved from the resins using 95% TFA and 5% water for 2 h. Following filtration the TFA was evaporated, and the peptides analyzed as described below.

Analysis of the Peptides

The crude peptides were analyzed by HPLC using a linear gradient of 5% to 50% solvent B (solvent A aqueous 0.1% TFA, and solvent B MeCN containing 0.1% TFA) over 45 min, at a flow rate of 1 mL/min, with monitoring at 214 nm (Table V). The molecular weights of these peptides were determined by ESI-MS.

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