Published in final edited form as:

Biopolymers. 2011 ; 96(1): 97–102. doi:10.1002/bip.21496.

Deletion of Ac-NMePhe¹ from [NMePhe¹]arodyn under Acidic Conditions: 1. Effects of Cleavage Conditions and N-Terminal Functionality

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Abstract

Peptides containing N-methylamino acids can exhibit improved pharmacodynamic and pharmacokinetic profiles compared to nonmethylated peptides, and therefore interest in these N-methylated peptides has been increasing in recent years. Arodyn (Ac[Phe^{1,2,3},Arg⁴,D-Ala⁸]Dyn A(1–11)-NH₂) is an acetylated dynorphin A (Dyn A) analog that is a potent and selective κ opioid receptor antagonist (Bennett *et al. J. Med. Chem.* **2002**, *45*, 5617), and its analog [NMePhe¹]arodyn shows even higher affinity and selectivity for κ opioid receptors (Bennett *et al., J. Pep. Res.* **2005**, *65*, 322). During the synthesis of [NMePhe¹]arodyn analogs, the arodyn (2–11) derivatives were obtained as major products. Analysis indicated that Ac-NMePhe was lost from the completed peptide sequence during acidic cleavage of the peptides from the resin and that the acetyl group played an important role in this side reaction. Different cleavage conditions were evaluated to minimize this side reaction and maximize the yield of pure [NMePhe¹]arodyn analogs. Modifications to the N-terminus of the peptides to prevent the side reaction were also explored. The incorporation of a heteroatom-containing group such as methoxycarbonyl as the N-terminal functionality prevented this side reaction, while the incorporation of a bulky acyl group could not. Substituting NMePhe with the conformationally constrained analog Tic (1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid) also prevented the side reaction.

Keywords

N-methylamino acid; dynorphin analogs; arodyn; side reaction

Introduction

There has been a significant increase recently in the study of N-methylated peptides.^{1–3} N-Methylated peptides have been isolated from a variety of natural sources including plants, marine animals and microorganisms.^{4,5} N-Methylation can increase metabolic stability towards peptidases,^{6,7} and elimination of the hydrogen bond donor can increase lipophilicity and thereby potentially increase bioavailability.⁸ Cyclosporines, natural products produced by the fungus *Beauveria nivea* that contain multiple N-methylamino acids, have anti-fungal, anti-inflammatory and immunosuppressive activities.^{2,9,10} Cyclosporine A, an undecapeptide with seven N-methylamino acids, was found to be orally

active,¹¹ even though it has a relatively high molecular weight, probably due to N-methylation and intramolecular hydrogen bonds. Therefore, N-methylated peptides are increasingly of interest as potential therapeutic agents. N-Methylamino acids are also of interest because of their conformational effects on peptides.^{1,6,7} The proportion of the *cis* amide bond conformation is significantly higher when the amide nitrogen is methylated,^{1,12,13} and elimination of a hydrogen bond donor can also affect the peptide secondary structure. More recently, N-methylated cyclic pentapeptides have been used as templates for the rational design of peptides with distinct backbone conformations.¹⁴

Our research focuses on the development of potent and highly selective peptide antagonists for kappa (κ) opioid receptors and examination of the structure-activity relationships (SAR) for antagonist activity at these receptors. In addition to their use as pharmacological tools, κ opioid receptor antagonists have the potential to be used therapeutically in the treatment of cocaine^{15,16} and opioid dependence^{17,18} and have been shown to have antidepressant¹⁹ and anti-anxiety^{20,21} activity. Dynorphin A (Dyn A, Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln) is thought to be an endogenous ligand for κ opioid receptors.²² Aroclon (Ac[Phe^{1,2,3},Arg⁴,D-Ala⁸]Dyn A(1–11)NH₂, **1**; Figure 1) is an acetylated Dyn A analog identified in our laboratory that is a potent and highly selective κ opioid receptor antagonist,²³ and its analog [NMePhe¹]aroclon (**2**; Figure 1) shows even higher affinity and selectivity for κ opioid receptors.²⁴

During the synthesis of **2**, two major products were obtained and characterized by high-performance liquid chromatography (HPLC) and electrospray ionization-mass spectrometry (ESI-MS). Mass spectral analysis of the mixture of products showed the desired peptide ($[M+2H]^{2+} = 774.9$) and another major product ($[M+2H]^{2+} = 673.8$) corresponding to aroclon(2–11) with the loss of the N-terminal Ac-NMePhe moiety (mass = 203.7) from the desired peptide. Cleavage of an aliquot of the peptide from the resin prior to acetylation indicated that the deletion was not due to incomplete coupling. Because of the remarkable pharmacological activity of [NMePhe¹]aroclon, this side reaction was studied in detail.

There have been reports of a similar side reaction involving trialkyl glycine derivatives in both linear and cyclic peptides,^{25–28} but there has been only one recent report of a similar side reaction involving an Ac-N-methyl- α -monoalkyl-amino acid.²⁹ The mechanistic aspects of this side reaction involving trialkyl glycine derivatives have been studied (Scheme 1).^{25,29} The carbonyl oxygen of the acetyl group acts as a nucleophile and attacks the adjacent carbonyl group to form a five-membered oxazolinium intermediate, releasing the rest of the peptide. For N-methylamino acids with an α -hydrogen, the oxazolinium intermediate can undergo tautomerization to form a stabilized aromatic ring (Scheme 1). Here we describe our efforts to optimize the cleavage conditions to improve the yields of peptides containing an N-terminal Ac-N-methylamino acid, and the synthesis of stable analogs that do not undergo this side reaction.

Results and discussion

[NMePhe¹,Trp³]aroclon (**3**, Table 1), was chosen as the parent compound for these studies since this peptide has strong UV absorbance at 280 nm, facilitating monitoring this side reaction. Pure **3** underwent degradation under standard cleavage conditions (2 h, Reagent B, trifluoroacetic acid (TFA):H₂O:phenol:triisopropylsilane (TIPS) = 88:5:5:2,³⁰ Figure 2). Within 2 h, the typical time used for cleavage of peptides from the resin, approximately 60% of the peptide was degraded. Almost all of the parent peptide had decomposed after 6 h at room temperature, with [Trp³]aroclon(2–11) as the major product obtained (Figure 2).

The yield of the desired peptide **3** following cleavage from the resin was only about 8% under standard cleavage conditions (Reagent B, 2 h at room temperature). Different cleavage cocktails at lower temperature (4 °C) were evaluated to minimize the side reaction and to maximize the yield of pure **3** (Table 2). Incomplete deprotection of the Arg side chain 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) protecting group was observed under all of the examined conditions, presumably due to the lower cleavage temperature and resulting slower deprotection reactions. Interestingly, cleavage of the peptide by pure TFA for 3 h gave the highest yield (44%) of **3** among the different cleavage conditions examined. Interestingly, the addition of water as a scavenger significantly decreased the amount of the desired peptide obtained, as determined by HPLC.

Since the acetyl group as well as the first amino acid (NMePhe) was proposed to be involved in the deletion reaction, we designed aroldyn analogs in which the acetyl group of **3** was either deleted (compound **4**) or replaced with an alternative group, or the NMePhe was substituted with Phe (compound **5**) or Tic (Tic = 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid, compound **6**, Table 1). (The structures of Phe, NMePhe, and Tic are compared in Figure 3.) Compounds **7–10** (Table 1) are acylated with heteroatom-containing groups, while peptides **11–17** (Table 1) are acylated with a variety of acids, some of which are quite sterically hindered. All of these analogs were synthesized using standard solid-phase peptide synthesis (SPPS) and cleaved using Reagent B at room temperature for 2 h. HPLC and ESI-MS analysis were used to identify the desired peptides and the deletion product. Retention times (t_R) of the corresponding desired peptides are shown in Table 1. The percent peak area in the HPLC chromatograms for the desired peptides and the (2–11) deletion products are shown in Figure 4.

As expected based on the mechanism, the des-acetyl analog **4** (Table 1) gave the expected peptide without any evidence of the deletion sequence (Figure 4), verifying the important role of the acetyl group in the deletion reaction. When the first amino acid residue is Phe (compound **5**) instead of NMePhe, no deletion product was detected. Thus the N-methyl group is required for the deletion reaction to occur, most likely due to changing the backbone conformation to one that favors the cyclization. When the first amino acid is the conformationally constrained NMePhe analog Tic (compound **6**), no deletion side product was detected (Figure 4). This suggests that the formation of the 5-membered ring intermediate does not occur for this cyclic N-alkyl amino acid.

Little to no deletion sequence was detected following cleavage of peptides **7–10** from the resin (Figure 4). Groups attached to the N-terminus that contain a heteroatom can decrease the electron density on the carbonyl and thus make the oxygen less nucleophilic, preventing the side reaction. An oxygen atom attached to or separated by one methylene from the carbonyl (peptides **7** and **8**, respectively) both have a similar effect, although a small portion of deletion product (8%) was observed for peptide **8**. The methoxyl group in **7** can act both as an electron-withdrawing group by field effect and an electron-donating group by resonance effect. In this situation, the field effect appears to outweigh the resonance effect. This is similar to one report where the carbamate group was not as a good hydrogen bond acceptor as a tertiary amide,³¹ presumably also due to the electron-withdrawing effect outweighing the resonance effect. When glycine was coupled to the N-terminus (peptide **9**), no deletion sequence was observed, most likely due to the electron-withdrawing effect of the protonated amine. When the amine was acetylated (peptide **10**), however, a small amount of the deletion sequence (10%) was observed, similar to the results for peptide **8**. This is most likely due to the decreased electron-withdrawing effect of the acetylated amine. This also demonstrates that the peptide is reasonably stable when the N-methylamino acid is not at the N-terminus of a peptide.

The acyl groups incorporated in peptides **11–17**, however, could not prevent the side reaction, and thus the deletion sequence was the major product found following cleavage of these peptides from the resin (Figure 4). The two most sterically hindered analogs, where the acetyl group was substituted with Piv (pivaloyl, 2,2,2-trimethylacetyl) and 2,6-dimethoxybenzoyl groups (compounds **13** and **17**, respectively) yielded similar amounts of the desired peptide (11% and 17%, respectively) as **3** (8%).

Conclusions

In conclusion, we have identified approaches to decrease and in some cases eliminate the side reaction involving deletion of an acetylated N-methylamino acid from the N-terminus of a peptide. In order to obtain higher yields of these peptides, alternative cleavage conditions were explored. While alternative cleavage conditions increased the yields of the full-length peptide, the deletion reaction still occurred for the acetylated peptides, decreasing the yield of the desired peptide. Substitution of the N-terminal acetyl group with different electron-withdrawing groups (for example, a methoxycarbonyl group or an amino acid) was found to prevent the side reaction. Other acyl groups, including those with bulky substituents, however, did not prevent the deletion reaction. The pharmacological effects of these substituents on κ opioid receptor affinity, selectivity and efficacy are reported in the accompanying manuscript.

Experimental section

Materials

All Fmoc-protected (Fmoc = 9-fluorenylmethoxycarbonyl) amino acids were purchased from Bachem (King of Prussia, PA), Calbiochem-Novabiochem (San Diego, CA), Applied Biosystems (Foster City, CA), or Peptides International (Louisville, KY). Fmoc-PAL-PEG-PS resin (PAL-PEG-PS = peptide amide linker-poly(ethylene glycol)-polystyrene) was purchased from Applied Biosystems. Benzotriazole-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) was purchased from Calbiochem-Novabiochem. Dichloromethane (DCM), *N,N*-diisopropylethylamine (DIEA), *N,N*-dimethylformamide (DMF), acetic acid, diethyl ether, acetonitrile, methanol, and TFA were purchased from Fisher Scientific (Hampton, NH). 1-Hydroxybenzotriazole (HOBt) and TIPS were purchased from Acros Chemical Co. (Pittsburgh, PA). All other chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI).

Peptide synthesis

All peptides were synthesized on the PAL-PEG-PS resin (0.16 or 0.24 mmol/g, 200 mg) by SPPS using Fmoc-protected amino acids according to standard procedures (Scheme 2). The resin was first swollen with 10 mL of DCM/DMF (1:1, 2 × 10 min). The desired Fmoc-protected amino acids were coupled to the growing peptide chain with *N,N*-diisopropylcarbodiimide (DIC) and HOBt (4/4 relative to the resin substitution) or PyBOP, HOBt, and DIEA (4/4/10 relative to the resin substitution) in DMF (2 mL) for 2 h. Completion of the coupling reactions was determined by the ninhydrin and/or the chloranil tests.^{32,33} The side chains of Lys and Trp were protected by Boc (*tert*-butoxycarbonyl), and the side chain of Arg by Pbf. Following the coupling reaction, the resin was washed with 10 mL of DCM/DMF (1:1, 5 × 30 sec). The Fmoc group was then removed using 20% piperidine in DMF (5 mL, 2 × 10 min), the resin washed with DCM/DMF (1:1, 10 × 30 sec) and the next amino acid then coupled to the resin. After completion of the peptide assembly and removal of the Fmoc group from the N-terminal residue, different acyl groups were attached to the N-terminal free amine, except for the peptides **4** and **9** which have a free amine at the N-terminus. For the synthesis of **3**, **5**, **6** and **10**, acetic anhydride (20 equiv) in

DMF (2 mL) was reacted with the resin for 30 min. The appropriate acid anhydrides (20 equiv in DMF) were used to synthesize **11–13**. The corresponding acid chlorides (20 equiv) with an equivalent amount of DIEA in DMF (2 mL) were used to prepare **7, 8, and 14–17**. The resins were then washed successively with DCM/DMF (1:1, 5 × 30 sec), and finally with methanol. The resins were dried under vacuum before cleavage.

Cleavage of the peptides from the resin under standard conditions

The peptides were cleaved from the resin by treating with Reagent B for 2 h.30 The solutions were then filtered from the resin and washed with TFA (1 mL). The solutions were diluted with 10% acetic acid (30 mL) and extracted with ethyl ether (2 × 30 mL), and the ether extracts then back extracted with acetic acid (2 × 10 mL). Residual ether in the combined aqueous solutions was evaporated under vacuum, and the solutions were lyophilized to give the crude peptides.

Cleavage of peptide 3 under different cleavage conditions at 4 °C

Protected peptide **3**-resin (80–110 mg) was mixed with 1 mL of one of three different cleavage reagents (pure TFA, Reagent B, or 95% TFA with 5% water) for 1 to 4 h at 4 °C, as shown in Table 2. Aliquots (0.2 mL) of the cleavage reactions were removed at various times and diluted with 2 mL water. After filtering, the filtrates were frozen, lyophilized, dissolved in water and analyzed by HPLC using a gradient of aqueous MeCN containing 0.1% TFA as described below.

Treatment of purified peptide 3 with Reagent B at room temperature

Purified peptide **3** (2.23 mg) was mixed with 1 mL of reagent B, and aliquots of the reaction mixture (0.1 mL) were removed at various time points and diluted with H₂O (1 mL). The aliquots were frozen, lyophilized, dissolved in water and analyzed by HPLC.

HPLC and ESI-MS analysis

All of the crude peptides were analyzed by HPLC. A linear gradient of 5–50% 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, was used for analysis. The yields of the desired peptides and deletion product were quantified using the absorbance at 214 nm. Peptide **3** has a retention time of 24.9 min, while the deletion product [Trp³]arodyn (2–11) has a retention time of about 15.4 min. The molecular weights of the peptides were determined by ESI-MS (Waters, Q-TOF analyzer).

Acknowledgments

The authors thank Sandra Bartlett at the University of Kansas for helpful suggestions on the research. The research was supported by grant R01DA018832 from the National Institute on Drug Abuse.

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Ac-R-Phe-Phe-Arg-Leu-Arg-Arg-D-Ala-Arg-Pro-Lys-NH₂

R = Phe Arodyn (**1**)

R= NMePhe [NMePhe¹]arodyn (**2**)

Figure 1.
Structures of arodyn (1) and [NMePhe¹]arodyn (2)

Figure 2.
Degradation of pure **3** in Reagent B at room temperature

Figure 3.
Structural comparison of Phe, NMePhe, and Tic

Figure 4. Percentage of desired peptide vs. deletion product obtained for peptide **3** and its analogs following cleavage of the peptides from the resin under standard conditions (Reagent B for 2 h at room temperature) as determined by HPLC.

(R = remainder of the peptide)

Scheme 1.

Proposed mechanism for the deletion of an Ac-N-methyl- α -monoalkyl-amino acid from the N-terminus of peptides under acidic conditions^{25,29}

Scheme 2.
SPPS of aroclon analogs

Table 1

Structures and retention times (t_R) of Peptide **3** and its analogs

X-NMePhe-Phe-Trp-Arg-Leu-Arg-Arg-D-Ala-Arg-Pro-Lys-NH₂

#	X	t_R (min) ^a	#	X	t_R (min) ^a	#	X	t_R (min) ^a
3	Ac	24.9	8	PhOCH ₂ CO	30.2	13	Piv	24.7
4	H	18.1	9	Gly	19.0	14	C ₆ H ₅ CH ₂ CO	30.1
5^b	Ac-Phe ¹	22.7	10	Ac-Gly	22.6	15	C ₆ H ₅ CO	29.3
6^b	Ac-Tic ¹	24.0	11	CH ₃ CH ₂ CO	ND ^c	16	2-MeO-C ₆ H ₄ CO	27.7
7	CH ₃ OCO	25.5	12	(CH ₃) ₂ CHCO	26.3	17	2,6-diMeO-C ₆ H ₃ CO	28.6

^aHPLC conditions: 5–50% acetonitrile containing 0.1% TFA over 45 min at 1 mL/min; the retention time of [Trp³]arodyn (2–11) is 15.4 min.

^bFirst amino acid is Phe or Tic instead of NMePhe.

^cNo desired peptide was observed under standard cleavage conditions.

Table 2

Effects of different cleavage conditions and reaction times at 4 °C on the yield of Peptide **3**, as measured by HPLC

Cleavage Cocktail	Cleavage Time (h)	% Area of 3
Reagent B ^a	2	17
	3.5	25
Pure TFA	1	37
	2	35
	3	44
	4	35
TFA: water = 95:5	1	8
	2	11
	3	22

^aTFA:phenol:water:TIPS (triisopropylsilane) = 88:5:5:2