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Synthesis and Conformational Analysis of a Cyclic Peptide Obtained via *i* to *i*+4 Intramolecular Side-Chain to Side-Chain Azide−Alkyne 1,3-Dipolar Cycloaddition

Sonia Cantel,[†] Alexandra Le Chevalier Isaad,[‡,§] Mario Scrima,[⊥] Jay J. Levy,[∥] Richard D. DiMarchi,[∥] Paolo Rovero,[‡,#] Jose A. Halperin,[†,▽] Anna Maria D'Ursi,[⊥] Anna Maria Papini,[‡,§] and Michael Chorev*[,†,▽]

Laboratory for Translational Research, Harvard Medical School, One Kendal Square, Building 600, Cambridge, Massachusetts 02139, Laboratory of Peptide & Protein Chemistry & Biology, University of Firenze, Dipartimento di Chimica Organica, Polo Scientifico e Tecnologico, University of Firenze, Via della Lastruccia 13, I-50019 Sesto Fiorentino, Italy, Department of Chemistry, Indiana University, 800 E. Kirkwood, Bloomington, Indiana 47405, Dipartimento di Scienze Farmaceutiche, Via Ponte Don Melillo 11C, Salerno, I-84084, Italy, Dipartimento di Scienze Farmaceutiche, University of Firenze, Polo Scientifico e Tecnologico, Via Ugo Schiff 3, I-50019 Sesto Fiorentino, Italy, and Department of Medicine, Brigham and Women's Hospital, 75 Francis Street, Boston, Massachusetts 02115

michael_chorev@hms.harvard.edu

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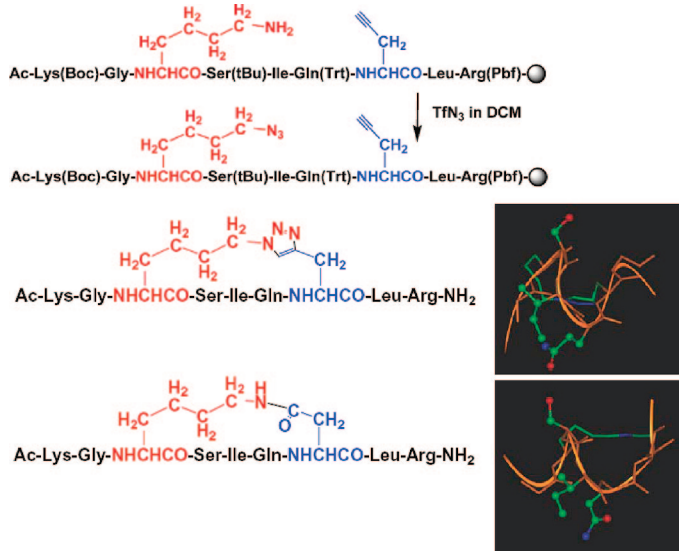

Intramolecular side-chain to side-chain cyclization is an established approach to achieve stabilization of specific conformations and a recognized strategy to improve resistance toward proteolytic degradation. To this end, cyclizations, which are bioisosteric to the lactam-type side-chain to side-chain modification and do not require orthogonal protection schemes, are of great interest. Herein, we report the employment of Cu^{I}-catalyzed 1,3-dipolar cycloaddition of side chains modified with azido and alkynyl functions and explore alternative synthetic routes to efficiently generate 1,4-disubstituted [1,2,3]triazolyl-containing cyclopeptides. The solid-phase assembly of the linear precursor

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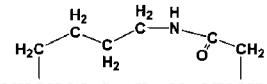
including ϵ-azido norleucine and the propargylglycine (Pra) in positions *i* and *i*+4, respectively, was accomplished by either subjecting the resin-bound peptide to selective on-resin diazo transformation of a Lys into the Nle(ϵ-N_3) or the incorporation of Fmoc-Nle(ϵ-N_3)-OH during the stepwise build-up of the resin-bound peptide **1b**. Solution-phase Cu^I-catalyzed 1,3-dipolar cycloaddition converts the linear precursor Ac-Lys-Gly-Nle(ϵ-N_3)-Ser-Ile-Gln-Pra-Leu-Arg-NH_2 (**2**) into the 1,4-disubstituted [1,2,3]triazolyl-containing cyclopeptide [Ac-Lys-Gly-Xaa(&[1])-Ser-Ile-Gln-Yaa(&[2])-Leu-Arg-NH_2][(&[1]$(CH_2)_4$-1,4-[1,2,3]triazolyl-CH_2&[2])] (**3**). The conformational preferences of the model cyclopeptide **3** (**III**), which is derived from the sequence of a highly helical and potent *i* to *i*+4 side-chain to side-chain lactam-containing antagonist of parathyroid hormone-related peptide (PTHrP), are compared to the corresponding lactam analogue Ac[Lys^{13}(&[1]),Asp^{17}(&[2])]hPTHrP(11−19)NH_2 (**II**). CD and NMR studies of **3** and **II** in water/hexafluoroacetone (HFA) (50:50, v/v) revealed a high prevalence of turn-helical structures involving in particular the cyclic regions of the molecule. Despite a slight difference of the backbone arrangement, the side-chains of Ser, Gln, and Ile located at the *i*+1 to *i*+3 of the ring-forming sequences share the same spatial orientation. Both cyclopeptides differ regarding the location of the turn-helical segment, which in **II** involves noncyclized residues while in **3** it overlaps with residues involved in the cyclic structure. Therefore, the synthetic accessibility and conformational similarity of *i* to *i*+4 side-chain to side-chain cyclopeptide containing the 1,4-disubstituted [1,2,3]triazolyl moiety to the lactam-type one may result in similar bioactivities.

Introduction

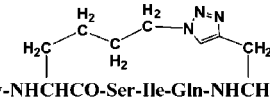
Intramolecular side-chain to side-chain cyclization of linear peptides has been employed to achieve rigidification that results in either restricting the conformational freedom and bias toward a specific ensemble of bioactive conformation and/or reducing susceptibility toward proteolytic enzymes, thus increasing the metabolic stability in vitro and more significantly in vivo. Cyclization in the form of disulfide bridge between two cysteines is the most abundant post-translational modification resulting in side-chain to side-chain cyclization. However, under certain redox potentials, the disulfide bridge will behave as a relatively transient modification yielding either the reduced linear form or generate a variety of intermolecular disulfide-containing products. On the other hand, the non-biogenic cyclization via lactam formation between side chains of ω-amino- and ω-carboxyl-α-amino acids or ring-closing metathesis are much more durable but require synthetic schemes that include either orthogonal protection to enable targeted side-chain to side-chain cyclizations or non-biogenic amino acids such as ω-alkenyl-containing amino acid building blocks.[1–4] Nevertheless, orthogonal sets of protecting groups such as Boc strategy with Bzl/Fmoc & OFm,[2] Boc strategy with Bzl & Trt/oxime resin/Pac,[5] or Fmoc strategy with Boc & tBu/Allyl & Alloc[6] enabled the synthesis of potent peptide lactams such as GnRH antagonist,[7] PTHrP agonist,[8] and an inhibitor of the HIV-1 Rev-RRE interaction.[9] To this end, development of new side-chain to side-chain cyclization modes, which are bioisosteric, hydrolytically and proteolytically stable, and simple to synthesize are of great interest.

SCHEME 1. (I) Linear parent Nonapeptide N^{α}Ac-PTHrP(11−19)NH_2 Derived from hPTHrP, (II) *i* to *i*+4 Side-Chain to Side-Chain Lactam Analogue of I, [Ac-Lys-Gly-Lys(&[1])-Ser-Ile-Gln-Asp(&[2])-Leu-Arg-NH_2], and (III) *i* to *i*+4 Side-Chain to Side-Chain 1,4-Disubstituted [1,2,3]Triazolyl-Bridged Analog of I [Ac-Lys-Gly-Xaa(&[1])-Ser-Ile-Gln-Yaa(&[2])-Leu-Arg-NH_2][(&[1]$(CH_2)_4$-1,4-[1,2,3]triazolyl-CH_2&[2])] (3)

I. Ac-Lys-Gly-Lys-Ser-Ile-Gln-Asp-Leu-Arg-NH_2



II. Ac-Lys-Gly-NHCHCO-Ser-Ile-Gln-NHCHCO-Leu-Arg-NH_2



III. Ac-Lys-Gly-NHCHCO-Ser-Ile-Gln-NHCHCO-Leu-Arg-NH_2

3

The recently introduced Cu^I-catalyzed azide−alkyne 1,3-dipolar Huisgen's cycloaddition[10,11] as a prototypic "Click reaction"[12] presented a promising opportunity to develop a new paradigm for intramolecular side-chain to side-chain cyclization in peptides. The inherent high energetics and chemical selectivity of the azide and alkynyl functions, the demonstrated bioor-

* To whom correspondance should be addressed. Tel: (617) 621-6145. Fax: (617) 621-6148.
† Laboratory for Translational Research, Harvard Medical School.
‡ Laboratory of Peptide & Protein Chemistry & Biology, University of Firenze.
§ Dipartimento di Chimica Organica, University of Firenze.
⊥ Dipartimento di Scienze Farmaceutiche.
|| Department of Chemistry, Indiana University.
Dipartimento di Scienze Farmaceutiche, University of Firenze.
▽ Department of Medicine, Brigham and Women's Hospital.

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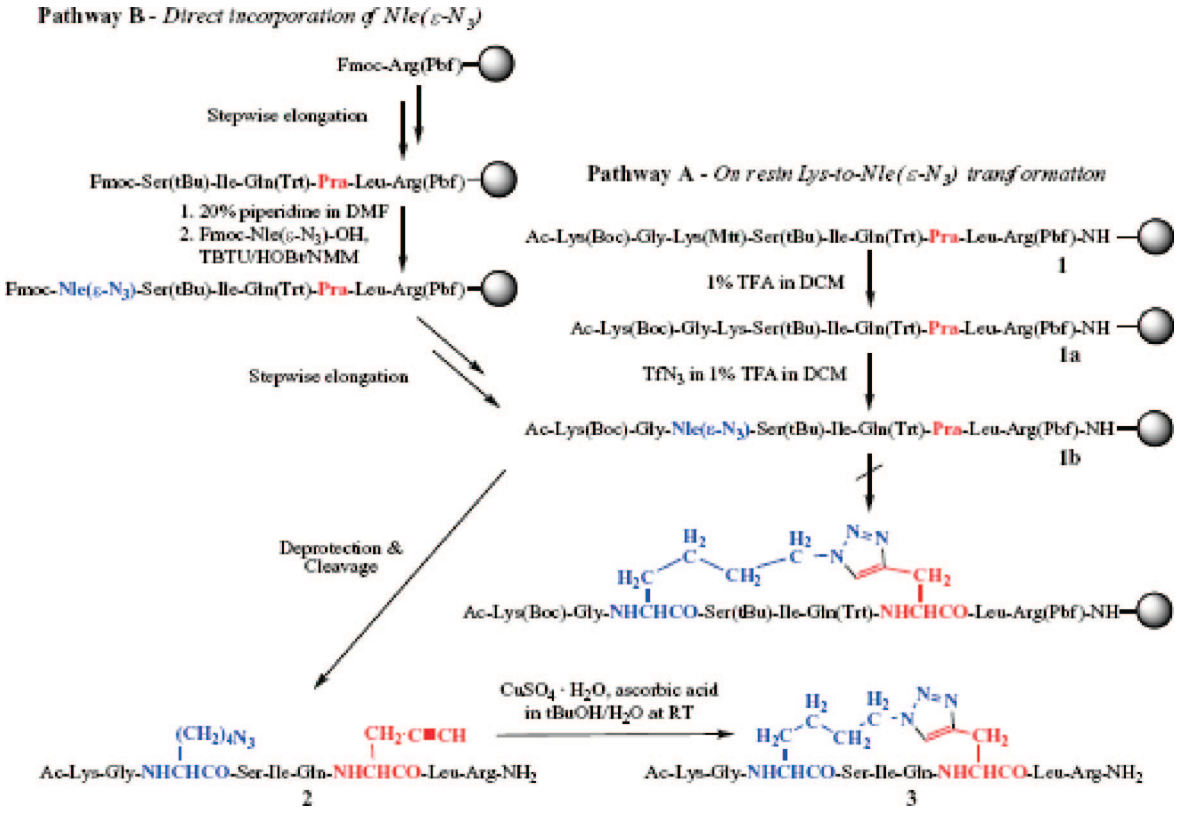
SCHEME 2. Strategies Employed in the Solid-Phase Synthesis of N^{α}Ac-[Xaa^{13}(&^{1}),Yaa^{17}(&^{2})]hPTHrP(11−19)NH_2]-[(&^{1}$(CH_2)_4$-1,4-[1,2,3]triazolyl-CH_2&^{2})] (3)[a]



[a] Pathway A: Stepwise assembly of the fully protected resin-bound peptide in which the ϵ-NH_2 of the two lysine residues are differently protected to allow selective deprotection and subsequent diazo-transfer reaction to on-resin transform Lys^{13} into Nle(ϵ-N_3). Pathway B: Stepwise on-resin assembly of the fully protected peptide **1b** incorporating Fmoc-Nle(ϵ-N_3)-OH as a building block. Cleavage and deprotection of **1b** obtained by either pathway generated the linear precursor **2** that was than cyclized by a Cu^I-catalyzed azide−alkyne 1,3-dipolar cycloaddition to yield the cyclic [1,2,3]triazolyl-containing peptide **3**.

thogonal characteristics of these functions,[13–19] the proteolytic stability,[20] and the peptidomimetic nature of the cycloaddition product, the 1,4-disubstituted [1,2,3]triazolyl moiety,[10,11,20–24] suggest that 1,4-disubstituted [1,2,3]triazolyl-mediated side-chain to side-chain cyclization could offer an interesting mode of structural constraint. Indeed, the 1,4-disubstituted [1,2,3]triazolyl serves as a rigid linking unit mimicking a *trans*-amide bond positioning the substituents in positions 1 and 4 5 Å apart, which is only slightly longer (5.1 Å) than the distance between two carbons separated by a *trans*-amide bond (3.9 Å). It has a slightly larger dipole moment (∼5 debye), which bisects the ring plane near atoms N3 and C5, and has the capacity of the N2 and N3 electron lone pairs to serve as hydrogen bond acceptors.[24] Incorporation of 1,4-disubstituted [1,2,3]triazolyl ϵ-amino acids as *trans*-amide dipeptide surrogates supported a variety of secondary structural elements such as α-helical coiled-coils,[22] a β-turn mimetic,[25] and an extended β-sheet-like hollow tubular assemblies.[26] Interestingly, a report focusing on the synthesis of a high capacity poly(ethylene glycol resin) and its application for peptide and organic syntheses mentions the synthesis of the only side-chain to side-chain 1,4-disubstituted [1,2,3]triazolyl-bridged peptide reported to date.[27] Stepwise incorporation of N^{α}-Fmoc-protected amino acids including 2-amino-3-azidopropionic and 2-aminopropargylic acids led to the i to i+5 side-chain to side-chain [1,2,3]triazolyl-bridged cyclic peptide.

Our study reports on the exploration of synthetic routes that may lead to an helix-mimetic cyclopeptide [Ac-Lys-Gly-Xaa(&^{1})-Ser-Ile-Gln-Yaa(&^{2})-Leu-Arg-NH_2][(&^{1}$(CH_2)_4$-1,4-

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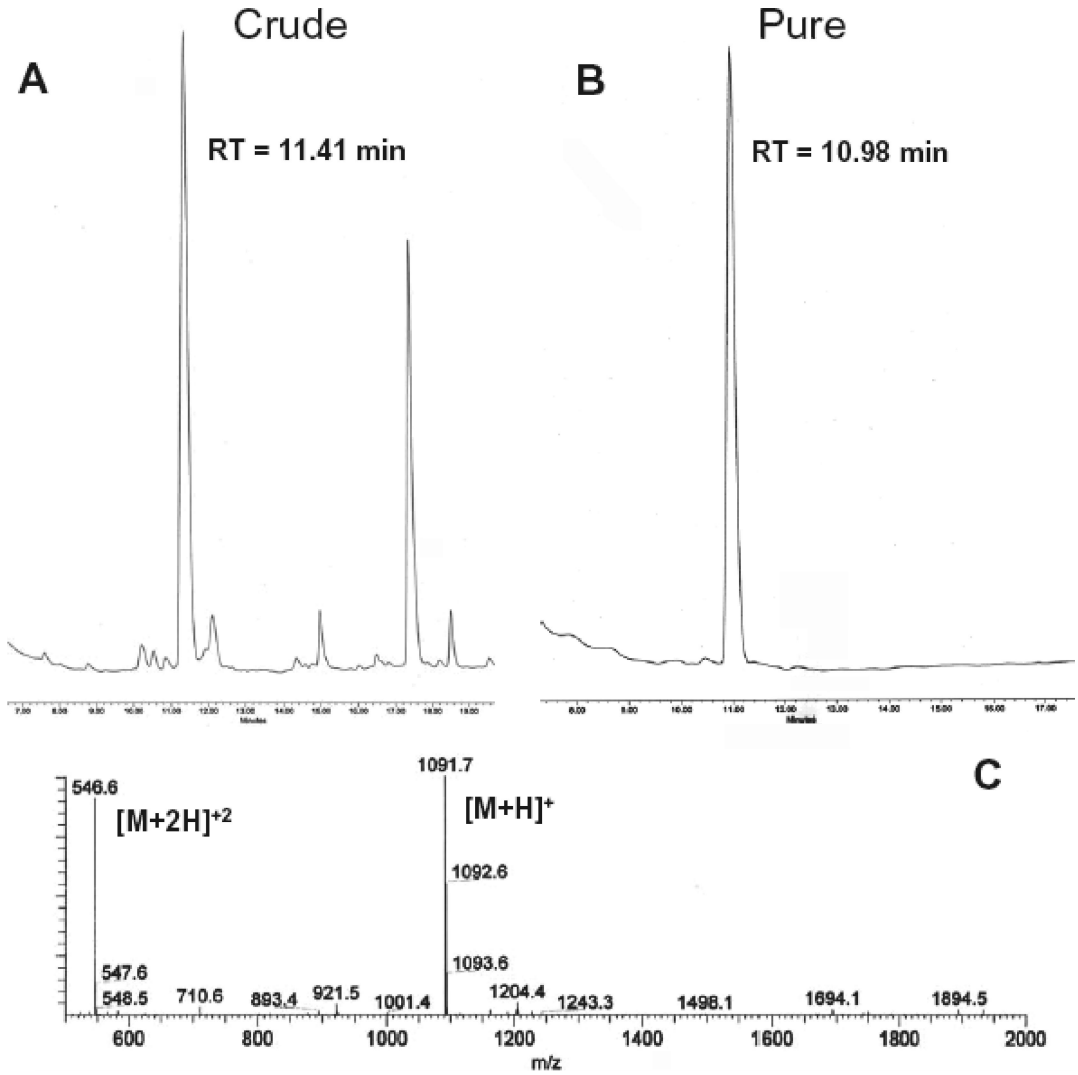

FIGURE 1. Linear precursor Ac[Nle^{13}(ϵ-N$_3$),Pra^{17}]hPTHrP(11−19)NH$_2$ (**2**). (A) Chromatogram of the crude mixture generated upon deprotection and cleavage from resin obtained following pathway A (Scheme 2). (B) Chromatogram of the purified linear precursor shown in panel A. (C) ESI-MS of the purified peak corresponding to t_R = 10.98 min shown in panel B. Observed are the mono- and diprotonated molecular ions of the linear precursor **2**.

[1,2,3]triazolyl-CH$_2$&^{2})]^{28} (Scheme 1, **III**), a model *i* to *i*+4 side-chain to side-chain 1,4-disubstituted [1,2,3]triazolyl-bridged peptide derived from a modified fragment of N$^{\alpha}$Ac-hPTHrP-(11−19)NH$_2$ (Scheme 1, **I**), and compares its solution conformation to the corresponding lactam analogue [Ac-Lys-Gly-Lys(&^{1})-Ser-Ile-Gln-Asp(&^{2})-Leu-Arg-NH$_2$] (Scheme 1, **II**). The parent cyclic *i* to *i*+4 side-chain to side-chain peptide [Lys^{13}(&^{1}),Asp^{17}(&^{2}),Tyr^{34}]hPTHrP(7−34)NH$_2$ from which this fragment was derived was previously reported to be a potent parathyroid hormone receptor 1 (PTHR1) antagonist containing an extended and stabilized α-helical conformation.[2,29]

Results and Discussion

Selection of Model System. The development and study of the new intramolecular side-chain to side-chain [1,2,3]triazolyl-containing modification was carried out in the context of a molecular model for which structural information on the closely related *i* to *i*+4 side-chain to side-chain lactam containing peptide is already available. To this end, we turned to the model cyclic peptide lactam N$^{\alpha}$Ac-[Lys^{13}(&^{1}),Asp^{17}(&^{2})]hPTHrP-(11−19)NH$_2$ that is a markedly trimmed version of the [Lys^{13}(&^{1}),-Asp^{17}(&^{2}),Tyr^{34}]hPTHrP(7−34)NH$_2$ (**II** in Scheme 1). In both peptides, a 20-membered lactam ring is formed by ring closure between the side-chain of Lys^{13} and the side-chain of Asp^{17}. The latter is a 5−10-fold more potent antagonist than the linear parent PTHrP antagonist,[2] and according to our previous CD, NMR, and molecular dynamic studies contains an extended and stabilized α-helical conformation (residues 13−18 and 22−32)[29] as compared with the linear parent peptide [Tyr^{34}]PTHrP-(7−34)NH$_2$. Therefore, synthesis of the newly developed intramolecular side-chain to side-chain [1,2,3]triazolyl-containing modification N$^{\alpha}$Ac-[Xaa^{13}(&^{1}),Yaa^{17}(&^{2})]hPTHrP(11−19)-NH$_2$][(&^{1}(CH$_2$)$_4$-1,4-[1,2,3]triazolyl-CH$_2$&^{2})] (**III** in Scheme 1) and the characterization of its conformational preferences in the context of the model lactam peptide N$^{\alpha}$Ac-[Lys^{13}(&^{1}), Asp^{17}-(&^{2})]hPTHrP(11−19)NH$_2$will provide insights on the relevance of this side-chain to side-chain modification as a transoid amide bond surrogate.

Synthetic Strategy. Both the relative ease of introduction of the azido and alkynyl functions into molecular entities and

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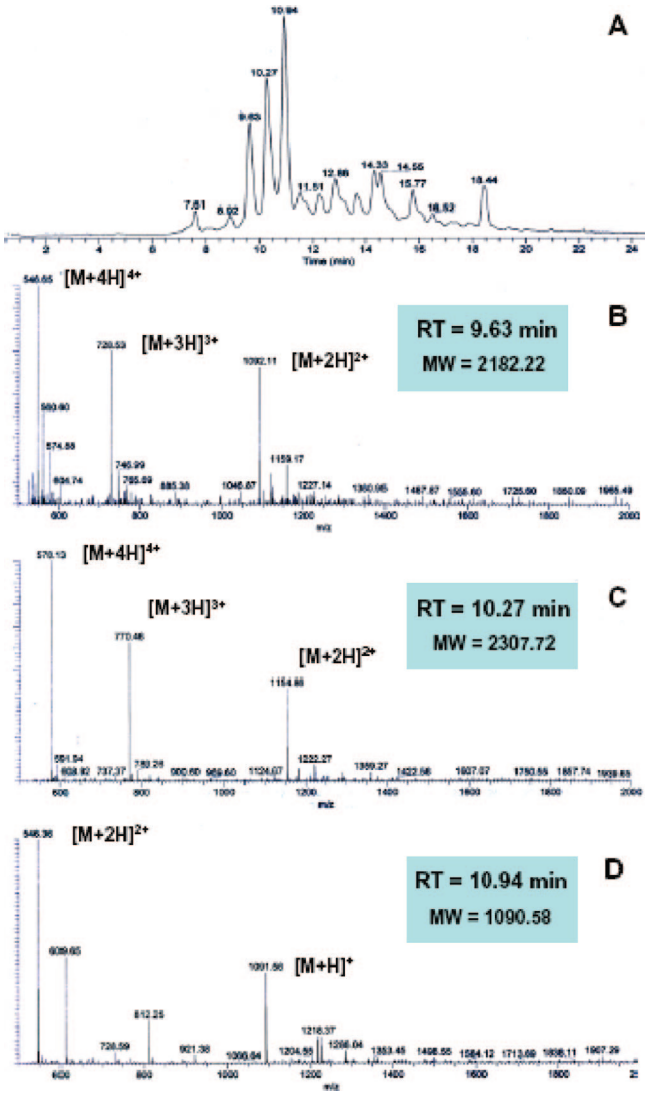

FIGURE 2. Analysis of the product mixture obtained from an attempt to carry out on-resin Cu^I-catalyzed azide−alkyne 1,3-dipolar cycloaddition on the resin-bound peptide **1b**. (A) Total Ion Current obtained from LC−ESI−MS of a product mixture obtained by deprotection and release of resin-bound linear precursor **1b** following treatment with CuI (2 equiv) and DIEA (50 equiv) in THF after ON incubation at rt. MS analyses identify the cyclodimer (t_R = 9.63 min) generating the corresponding di-, tri-, and tetraprotonated species (B), monoiodinated cyclodimer (t_R = 10.27 min) generating the corresponding di-, tri-, and tetraprotonated species (C), and the linear precursor **2** (t_R = 10.94 min) generating the mono- and diprotonated molecular ions (D).

their apparent chemical inertness toward a wide range of reaction conditions (e.g., molecular oxygen and water) and reactive groups such as nucleophiles and electrophiles are well documented.[12,14,30] Therefore, our initial synthetic strategy was to develop a solid-phase synthesis of side-chain to side-chain [1,2,3]triazole-containing cyclic peptides by assembling the azido and alkynyl side-chains containing peptide and cyclizing the resin-bound peptide through Cu^I-catalyzed azide−alkyne 1,3-dipolar cycloaddition. In addition, we planned to follow two different strategies to introduce the L-2-amino-6-azidohexanoic acid residue [Nle(ϵ-N_3)] into the resin-bound peptide. While in pathway A the Nle(ϵ-N_3) residue is formed via a diazo-transfer reaction on the selectively deprotected ϵ-amino moiety of the otherwise fully protected resin-bound peptide, in pathway B, Fmoc-Nle(ϵ-N_3)-OH is incorporated as part of the standard stepwise on-resin peptide assembly involving repetitive cycles of coupling and deprotection steps (Scheme 2).

Solid Phase Assembly of Peptide and Click Reaction. The assembly of the resin-bound peptides **1a** and **1b** by either DEPBT/DIEA-[31,32] or TBTU/HOBt/NMM-mediated[33] couplings in pathways A and B, respectively, was carried out in a straightforward manner. The application of Boc and Mtt for protecting the ϵ-amino functions on Lys^{11} and Lys^{13}, respectively, enabled selective cleavage of the highly acid labile Mtt by 1% TFA in DCM without compromising the resin anchorage and the other side-chain protecting groups. On-resin diazo-transfer of the ϵ-amino on Lys^{13} to the azido function following previously published procedure used to transform α-amino- into azido functions either on resin[34,35] or in solution[36] and yielded **1b** (Scheme 2, pathway A). Deprotection of all side-chain protecting groups and simultaneous cleavage of the resin-bound peptide yielded the crude linear peptide **2** (Figure 1, panel A). Absence of peaks corresponding to the Lys^{13} containing linear peptide in the chromatogram and the mass spectrum (not shown, t_R = 9.53 min; m/z = 1065.65 or 533.32 corresponding to $[M + H]^+$ and $[M + 2H]^{+2}$, respectively) suggests a complete transformation of the Lys to the Nle(ϵ-N_3).

All of our attempts to carry out on-resin intrachain Cu^I-catalyzed side-chain to side-chain azide−alkyne 1,3-dipolar cycloaddition employing a wide range of reported conditions failed. We did not observe any click reaction in the presence of Cu^I (2 equiv)/ascorbic acid (2 equiv)/pyridine (10 equiv) at rt in solvent systems such as DMF/H_2O (9:1, ON), THF (2 days), or DCM (1 week) or after ON incubation at rt in the presence of CuI (0.5 equiv)/ascorbic acid (1 equiv)/2,6-lutidine (2 equiv). After 3 days at rt in the presence of $CuSO_4$ (100 equiv) and ascorbic acid (100 equiv) in DMSO/H_2O (8:2), we observed the appearance of a more hydrophobic peak with m/z similar to the starting material. Moreover, exposure of resin-bound peptide **1b** to CuI (2 equiv)/DIEA (50 equiv) in THF at rt ON led to side reactions that cause cyclodimerization and incorporation of iodine into some of the starting resin-bound parent peptide (Figure 2). Similar results were observed when CuBr replaced CuI. Heating **1b** ON at 110 °C in toluene in the presence of CuBr (100 equiv)/DBU (3 equiv) also resulted in partial conversion to the corresponding putative cyclodimer. Interestingly, Punna and co-workers in their attempt to carry out on-resin side-chain (Pra) to end-group (5-azidopentanoyl) macrocyclization through Cu^I-catalyzed azide−alkyne cycloaddition reaction reported ~60% yield of cyclodimerization.[37,38] Reaction conditions included CuI (0.5 equiv), Na ascorbate (1 equiv), 2,6-lutidine (2 equiv), and CH_3CN/DMSO/H_2O (8:2:1) for 16 h at rt. They suggest that this propensity for selective dimerization is driven by interchain π-complexation across adjacent Cu-acetylide centers, steric hindrance, and crowding as the causes

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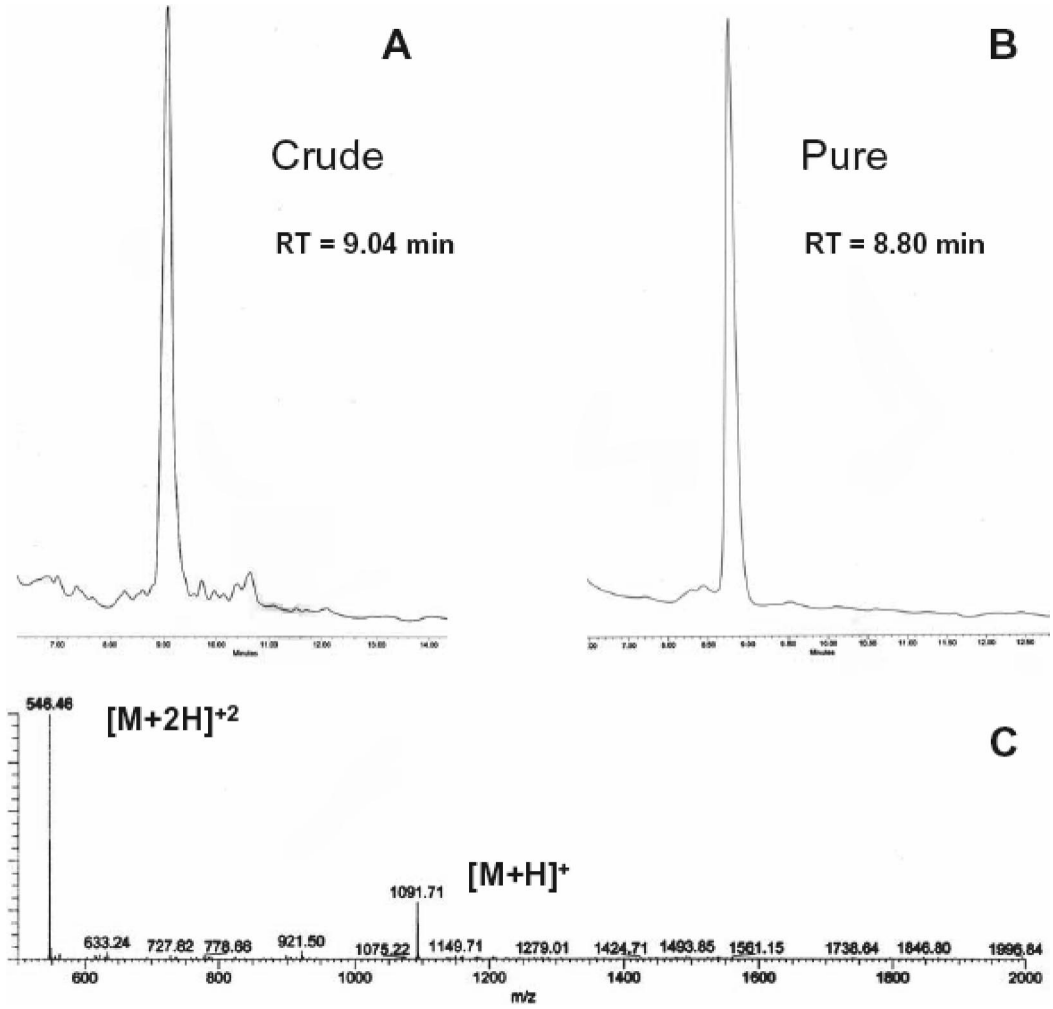

FIGURE 3. Cyclic Ac[Xaa^{13}(&^{1}),Yaa^{17}(&^{2})]hPTHrP(11−19)NH_2][(&^{1}$(CH_2)_4$-1,4-[1,2,3]triazolyl-CH_2&^{2})] (**3**). (A) Chromatogram of the crude mixture obtained upon solution-phase Cu^I-catalyzed azide−alkyne 1,3-dipolar cycloaddition carried out on the linear precursor **2** (Scheme 2). (B) Chromatogram of the purified [1,2,3]triazolyl-containing cyclopeptide **3** shown in panel A. (C) ESI-MS of the purified peak corresponding to t_R = 8.80 min shown in panel B. Observed are the mono- and diprotonated molecular ions of the cyclopeptide **3**.

for preferential cyclodimerization. The formation of 5-iodo[1,2,3]-triazolyl-containing cyclic tetrapeptide (3−42% yield) during Cu^I-catalyzed azide−alkyne cycloaddition was reported by Bock and co-workers during their CuI-mediated head-to-tail cyclization of a tetrapeptide analogue in the presence of either 2,6-lutidine/DIPEA or DBU.[39] Taken together, the propensity for on-resin cyclodimerization observed by us in our side-chain to side-chain cyclization concurs with previously reported head-to-tail cyclization studies in which the distance between the azido and alkynyl functions was either much greater[37] or significantly shorter[38] than in our peptide.

Deprotection of the linear resin-bound peptide **1b** with concomitant cleavage from resin yielded the crude linear precursor **2** (Figure 1, panel A), which was subjected, after purification by HPLC, to solution-phase intrachain Cu^I-catalyzed side-chain to side-chain azide−alkyne 1,3-dipolar cycloaddition. Complete conversion of the linear precursor **2** to the desired cyclic [1,2,3]triazolyl-containing peptide **3** was achieved after ON incubation at rt in tBuOH/H_2O (1:2 v/v) in the presence of 4.4-fold molar excess of $CuSO_4 \cdot 5H_2O$ and ascorbic acid (Figure 3). Importantly, attempts to accomplish this cyclization under conditions such as CuI and DIEA or 2,6-lutidine ON in dry acetonitrile (10 equiv and 100 equiv, respectively) or CuBr in the presence of DBU (10 equiv and 5 equiv, respectively) in toluene ON at 110 °C failed to generate the desired click product.

The lack of cyclodimerization observed in our *i* to *i*+4 side-chain to side-chain azide−alkyne 1,3-dipolar cycloaddition (closing 21-member ring) in the solution phase was also reported for the solution-phase Cu^I-mediated head-to-tail cyclization of N-terminal-azido and C-terminal-alkynyl substituted tetrapeptide analogues carried out in the presence of different Cu salts, additives, and solvents, and at different temperatures (closing 13- or 14-membered rings).[24,] These observations differ from other solution-phase cyclization studies of head-to-tail azide-to-alkyne 1,3-dipolar cycloaddition that reported high propensity for cyclodimerization.[25,40–42] Regardless of the nature of the linear precursor, whether it was a trisaccharide,[40] carbohydrate/peptide hybrid,[41] dipeptide-containing peptidomimetic,[25] or macrocycles of diverse nature,[42] cyclodimerization was observed. Evidently, further studies will be needed to better

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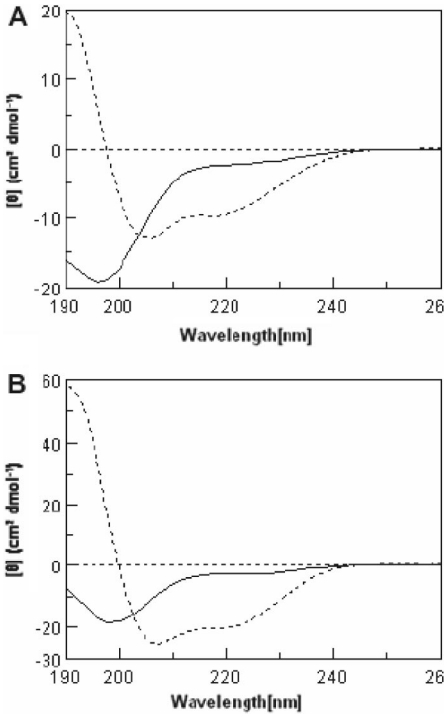

FIGURE 4. CD spectra of lactam- (A) and [1,2,3]triazolyl-containing cyclopeptide (B) in water (solid line) and water/HFA (50:50, v/v) (broken line) at 300 K.

understand the relationship between the chemical nature of the linear precursor, the distance and structural presentation of the azido and the alkynyl moieties, and the nature of the reagent and reaction conditions used in the Cu^I-catalyzed azide−alkyne 1,3-dipolar cycloaddition leading to intramolecular cyclization vs. cyclodimerization.

Importantly, in the absence of the catalyst Cu^I we did not observe any spontaneous cyclization or cyclodimerization of the resin-bound linear precursor **1b** either after 2 months of storage at 4 °C in the absence of solvent or for 3 weeks at rt in DCM. Neither spontaneous cyclization/cyclodimerization nor decomposition of the linear precursor **2** was observed after 3 days of incubation in 50 mM phosphate buffer (pH 7.4) at rt. These observations underscore that the azido−alkynyl 1,3-dipolar cycloaddition is catalyst/additive dependent and that the apparent infinite concentration of the [1,2,3]triazole forming functions is insufficient to overcome the energetic barrier that prohibits spontaneous intramolecular cyclization.

CD and NMR Analysis. Protocols for CD and NMR analysis are included in the Supporting Information. A preliminary screening of the conformational preferences of lactam- and [1,2,3]triazolyl-containing peptides as a function of the environment was performed by means of CD spectroscopy. For comparison CD spectra of the linear parent fragment of N^{α}Ac-hPTHrP(11−19)NH_2 were recorded in the same solution conditions. CD spectra were recorded in water solution and in a mixture of 50/50 v:v water/HFA mixture.

TABLE 1. ^{1}H Chemical Shift Assignments for [1,2,3]Triazolyl-Containing Cyclopeptide 3 and Lactam Cyclopeptide II in Water at 600 MHz and 300 K[a]

	[1,2,3]Triazolyl-Containing Cyclopeptide **3**						
residue	NH	$C^{\alpha}H$	$C^{\beta}H$	$C^{\gamma}H$	$C^{\delta}H$	others	Δα
Lys[11]	8.379	4.152	Hβ2 1.737 Hβ3 1.662	Qγ 1.357	Qδ 1.586	Qε 2.977	−0.208
Gly[12]							
Xaa[13]	8.148	4.197	Hβ2 1.835 Hβ3 1.762	Qγ 1.572	Qδ 1.639	Qε 3.202	−0.163
Ser[14]							
Ile[15]	7.432	3.968	1.780	Hγ12 1.342 Hγ13 1.113 Qγ2 0.817	Qδ1 0.751		−0.018
Gln[16]	7.978	4.023	Qβ 2.009	Qγ 2.316		Qε2 6.855	−0.347
Yaa[17]	8.234	4.426	Hβ2 2.885 Hβ3 2.801				−0.334
Leu[18]	7.970	4.242	Qβ 1.674	1.553	Qδ1 0.851 Qδ2 0.780		−0.072
Arg[19]							
	Lactam Cyclopeptide **II**						
Lys[11]	8.320	4.101					−0.259
Gly[12]	8.426	Hα1 3.862 Hα2 3.774					−0.108 −0.196
Lys[13]	7.957	3.469	Hβ2 1.731 Hβ3 1.492	Qγ 1.157	Hδ2 1.415 Hδ3 1.329	Qε 2.605	−0.891
Ser[14]	8.278	4.368	Qβ 3.754				−0.132
Ile[15]	7.392	3.930	1.773	Hγ12 1.393 Hγ13 1.111 Qγ2 0.808	Qδ1 0.732		−0.020
Gln[16]	7.958	3.937	Qβ 1.942	Qγ 2.274			−0.433
Asp[17]	8.160	4.611	Hβ2 2.816 Hβ3 2.568				−0.149
Leu[18]	7.582	4.180	Qβ 1.756	1.520	QQδ 0.797		−0.010
Arg[19]	7.931	4.189	Hβ2 1.804 Hβ3 1.719	Qγ 1.540	3.104		−0.191

[a] All values are referred to water residue signal. In Δα column, the difference between the $C^{\alpha}H$ chemical shifts typical of the random coil conformations and those relative to our cyclopeptides **3** and **II** are reported.

TABLE 2. ^{1}H Chemical Shift Assignments for [1,2,3]Triazolyl-Containing Cyclopeptide 3 and Lactam Cyclopeptide II in Water/HFA (50:50, v/v) at 600 MHz and 300 K[a]

residue	NH	C$^\alpha$H	C$^\beta$H	C$^\gamma$H	C$^\delta$H	others	Δα
				[1,2,3]Triazolyl-Containing Cyclopeptide **3**			
Lys^{11}							
Gly^{12}	7.260	3.376/3.275					−0.644
Xaa^{13}	7.276	3.519	1.228/1.137	0.795/0.840	0.910/0.920	Hζ 6.968 Hε 2.200/2310	−0.841
Ser^{14}	7.552	4.077	2.914/2.780				−0.423
Ile^{15}	7.049	3.230	1.319	1.037/0.595	0.231/0.236		−0.72
Gln^{16}	7.141	3.455	1.621/1.625	1.938/1.877		Hε 5.873/6.360	−0.915
Yaa^{17}	6.794	3.511	1.123/1.098				−1.247
Leu^{18}	7.382	3.706	1.348/1.261	1.074	0.230/0.245		−0.464
Arg^{19}	7.544	3.669	1.427/1.327	1.271/1.127	2.210/2.230	HH2 6.310/6450 Hε 6.349	−0.711
				Lactam Cyclopeptide **II**			
Lys^{11}	7.483	3.766	1.427	1.111 1.049	1.333		−0.594
Gly^{12}	7.760	3.547 3.504					−0.445
Lys^{13}	7.150	3.846	1.478 1.226	0.831	1.074	Hζ 6.989/7.001	−0.514
Ser^{14}	7.867	3.895	4.133 3.343				−0.605
Ile^{15}	7.324	3.532	1.54	Hγ1 1.199/0.837 Hγ2 0.230/0.345	Hδ1 0.421/0.403		−0.418
Gln^{16}	7.824	3.62	1.324/1.420	1.989/2.001			−0.75
Asp^{17}	7.869	4.362	2.495 2.340				−0.398
Leu^{18}	7.416	3.949	1.517 1.415	1.266	Hδ2 0.510/0.523	HH1 6.588	−0.221
Arg^{19}	7.436	3.899	1.578 1.394	1.100/1.203	2.321/2.422	HH2 6.201/6.302	−0.481

[a] All values are referred to water residue signal. In Δα column the difference between the C$^\alpha$H chemical shifts typical of the random coil conformations and those relative to our cyclopeptides **3** and **II** are reported.

Water is considered the most biocompatible medium to perform solution conformational studies since it is the main component of biological compartments. In contrast, water solutions enhance the conformational flexibility of short peptides compromising the collection of a sufficient number of NOE-based interprotonic distances needed for 3D-model building. Therefore, solvent mixtures with a viscosity higher than that of pure water are frequently used, in order to favor the prevalence of ordered, compact conformers over extended and/or disordered ones. Mixtures of water with alcohols, and mixtures of water with fluorinated organic solvent−water/trifluoroethanol (TFE), water/HFA, and water/hexafluoroisopropanol (HFIP)−are frequently used as media to induce "environmental constraints" in peptides. Fluorinated cosolvents contribute to a helix inducing/stabilizing effect, which does not override the intrinsic conformational tendency dictated by the specific sequence. [43–46]

Figure 4 shows a comparison of the CD spectra of lactam- and [1,2,3]triazolyl-containing peptides in water and in HFA/water (50/50 v/v). All the CD spectra recorded in water (pH 6.6) presented a negative band at 201 nm that is characteristic of a random coil structure, whereas the CD spectra recorded in water/HFA (50:50, v/v) mixtures,[47] show the well-shaped negative bands at 208 and 222 nm and an additional positive band at 192 nm typical of turn-helical structures. A single value deconvolution method[47] estimates for the linear N$^\alpha$Ac-hPTHrP(11−19)NH$_2$ peptide and for the two cyclopeptides a global turn-helical content greater than 50%.

NMR spectra were acquired in water and water/HFA (50:50, v/v) the same solvent mixture used for the CD measurement. To exclude potential aggregation, we recorded the 1D proton spectra of the cyclopeptides at a concentration range spanning 1−0.1 mM. At a peptide concentration of 0.1 mM, the peptides did not display any noticeable effects of aggregation. Chemical shift assignments of the proton spectra of both cyclopeptides in water and in water/HFA mixture were achieved via the standard systematic application of DQF-COSY,[48] TOCSY,[49] and NOESY[50] experiments, using the SPARKY software package according to the procedure of Wüthrich.[51]

The values of the proton chemical shifts of the lactam- and [1,2,3]triazolyl-containing peptides in water and in water/HFA (50:50, v/v) are reported in Table 1 and in Table 2. The chemical shifts differences between Hα values of peptides **II** and **3** analyzed by us, and those reported for random coil conformation are also reported in Table 1 and in Table 2. The analysis of chemical shifts differences of both the lactam- and [1,2,3]triazolyl-containing cyclopeptides in water and water/HFA mixture evidence that in water/HFA, but not in water solution, a remarkable upfield Hα chemical shifts is present as compared

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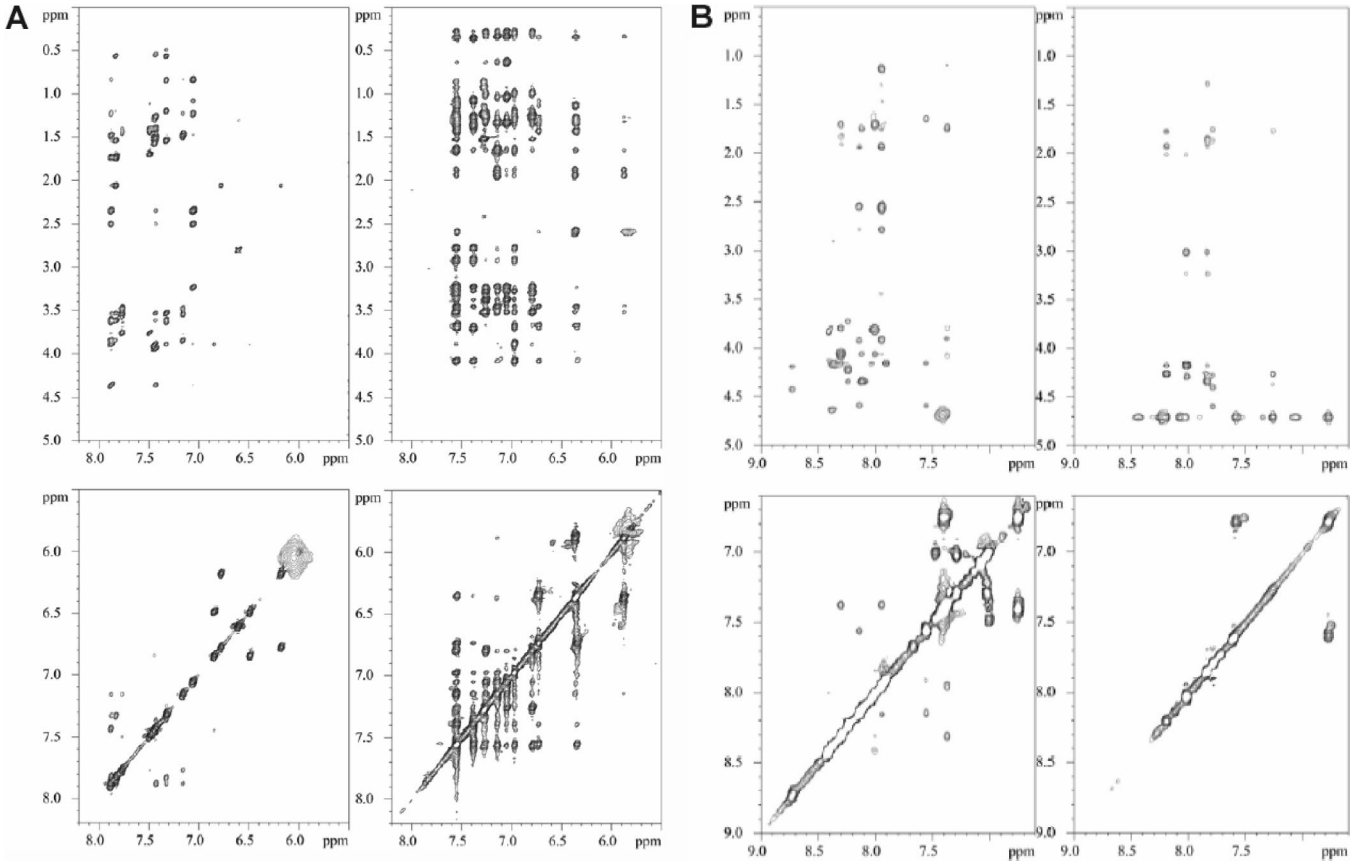

FIGURE 5. (A) Fingerprint region (top) and amide region (bottom) of the NOESY spectra of lactam- (left) and [1,2,3]triazolyl-containing (right) cyclopeptides in HFA/water (50:50, v/v) (600 MHz; $T = 300$ K, 1.8 mM). (B) Fingerprint region (top) and amide region (bottom) of the NOESY spectra of lactam- (left) and [1,2,3]triazolyl-containing (right) cyclopeptides in water (600 MHz; $T = 300$ K, 1.8 mM).

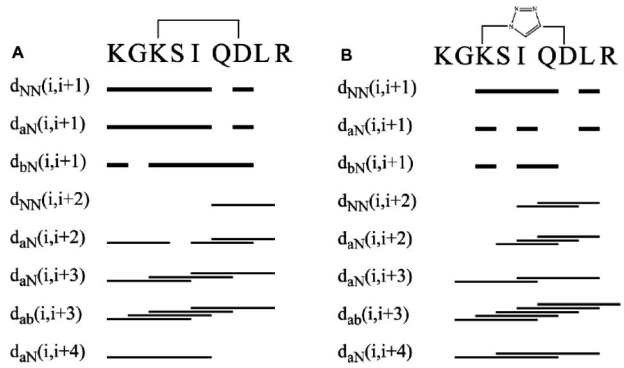

FIGURE 6. Sequential and medium-range NOEs for lactam- (A) and [1,2,3]triazolyl-containing (B) cyclopeptides. Data were obtained from a 600 MHz NOESY experiments with a mixing time of 200 ms and collected in water/HFA (50:50, v/v) at 300 K.

to the standard values reported for random coil conformations. The chemical shifts data, according to the ternary chemical shift index (CSI),[52] confirms the CD data on the prevalence of peptides **II** and **3** assuming in water/HFA solution turn-helix conformations whereas flexible random coil structures are evident in neat water solution.

Figure 5 shows the comparison of the amide and fingerprint regions of NOESY spectra of cyclopeptides collected in water and water/HFA (50:50, v/v). According to the CD data, the NOESY spectra of the biomolecules in water evidence few and weak NOE effects, confirming the ability of water to favor flexible and unfolded peptide conformations. On the contrary, a high number of well resolved cross-peaks are observable in water/HFA NOESY spectra, indicating the ability of the mixture to slow down the isoenergetic conformational equilibria, favoring the more ordered ones.

The diagnostic sequential and medium-range connectivities of both cyclopeptides in water/HFA are summarized in Figure 6. Whereas the ring forming region displays dNN(i, i+1) sequential connectivities and numerous dαN(i, i+3), dαβ(i, i+3), and dαN(i, i+4) medium range connectivities typical of a well structured domain, the terminal residues displayed only several sequential αCHi-NHi+1 NOEs consistent with a mixture of poorly structured or flexible regions.

NMR Structure Calculations. Three-dimensional structures of lactam- and [1,2,3]triazolyl-containing peptides **II** and **3** were calculated by simulated annealing procedures based on sequential and medium-range NOE-derived restraints (for details see Supporting Information). To avoid overestimation of NOE effects, NOESY spectra were collected using 50, 100, 150, and 200 ms mixing times. Interprotonic distances were derived from 100 ms mixing time NOESY spectrum. The best 20 structures out of 50 calculated were chosen according to the lowest values of the penalty (f) for the target function.[53] These structures were

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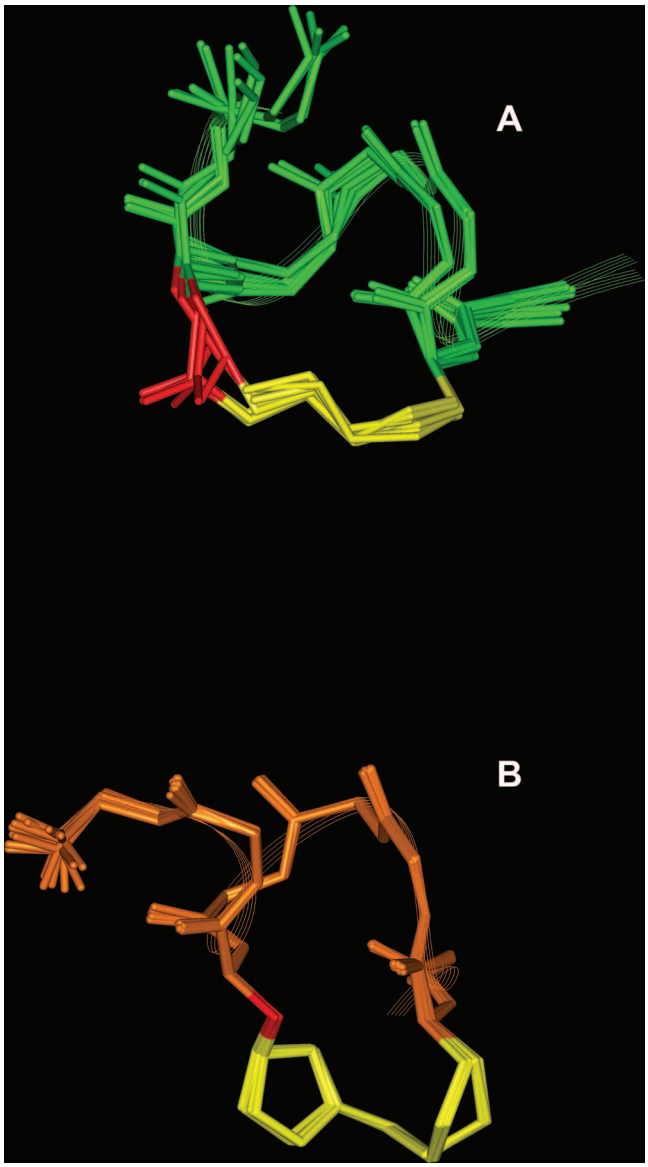

FIGURE 7. Best 20 calculated structures of lactam- (top) and [1,2,3]triazolyl-containing cyclopeptide (bottom) as derived from DYANA calculations. The structures are fitted on the heavy backbone atoms of the Lys^{13} and Asp^{17} included in the cyclic portion. Backbone atoms of lactam- and [1,2,3]triazolyl-containing cyclopeptides are colored in green and orange, respectively. The atoms responsible of the cyclization are colored in yellow.

energy minimized using the distance restraints with a progressively smaller force constant. The minimization procedure yielded an improved helical geometry and a lower total energy of the structures. To validate the resulting structures the PDB files were submitted to online PROCHECK software.[54]

Figure 7 shows an overlap of the 20 best structures of the lactam- and the [1,2,3]triazolyl-containing peptides, calculated by DYANA and then minimized with the SANDER module of the AMBER 5 software package. In both sets of structures a good overlap is observed in residues within the cyclic regions of the peptides. In these regions, the backbone rmsd was 0.57 Å and 0.62 Å for the lactam- and the [1,2,3]triazolyl-containing peptides, respectively. The backbone dihedral angles relative

TABLE 3. Values of the ϕ and Ψ Dihedral Angles of Cyclopeptides II and 3, respectively, As Derived from the Lowest Energy NMR Structures in Water/HFA

residue	cyclopeptides	ϕ	Ψ
Lys^{11}	**II**		−63.9
	3		−80.7
Gly^{12}	**II**	−71.2	97.2
	3	−89.4	61.8
Xaa^{13}	**II**	58.6	67.5
	3	60.8	−83.4
Ser^{14}	**II**	153.8	−84
	3	−66.3	−21.3
Ile^{15}	**II**	−70.7	−29.3
	3	−83.9	−30.0
Gln^{16}	**II**	−54.6	−51.2
	3	−156.7	−39.8
Yaa^{17}	**II**	−95.0	84.8
	3	−58.9	−35.6
Leu^{18}	**II**	−157.7	−67.5
	3	−62.4	−51.3
Arg^{19}	**II**	−142.5	
	3	−81.1	

the conformers included in the bundles are reported in Table 3. The structure bundle relative to the lactam-containing cyclopeptide **II** shows that the conformational preferences of this derivative are consistent with the presence of α-helical structure including the residues 11−17, and type I β-turn including the residues 12−15, 13−16, and 15−18. The cluster of NMR structures relative to the [1,2,3]triazolyl-containing cyclopeptide **3** shows that the conformational preferences of this derivative are consistent with the presence of α-helical structure at level of 14−19 and type I β-turn structure involving residues 16−19.

The comparison of the most representative NMR structures relative to lactam- and [1,2,3]triazolyl-containing cyclopeptide **II** and **3** shows that both peptides assume an α-helical structure in the cyclic part of the molecules. The structures of the two peptides differ regarding the location of the turn-helical segment, which in **II** involves noncyclized residues while in **3** it overlaps with residues involved in the cyclic structure. A careful inspection of the cyclic portions shows that, despite a slight difference of the backbone arrangement, the side-chains are characterized by a common orientation, in particular Ser, Gln, and Ile located at the $i+1$ to $i+3$ positions of the ring forming sequence (Figure 8). This common spatial orientation of the side-chains of residues contained within the ring suggest that they will share a common interaction with the same macromolecular target and, if critical for biological activity, they will also induce similar biological response. Evidently, this assumption can not be tested with the current model peptide that lacks the N-terminal portion required for PTH receptor recognition and induction of intracellular signaling.

Experimental Section

Solid-Phase Synthesis of Ac[Lys^{13}(&^{1}),Asp^{17}(&^{2})]hPTHrP-(11−19)NH_2 (II). The fully protected resin-bound peptide was synthesized using an Fmoc-Arg(N^G-Pbf)-Rink amide MBHA resin (0.2 mmol, 0.7 mmol/g, 285 mg) on ABI 430A peptide synthesizer employing Boc/Bzl chemistry, in situ neutralization as reported by Schnolzer et al.[55] and preactivated HBTU couplings [2 mmol of N^{α}-Boc-amino acid, 1.9 mmol of HBTU (3.8 mL of 0.5 M in DMF), 1 mL of DIEA]. Side-chain protection of Asp^{17} and Lys^{13} consisted of Fm and Fmoc groups, respectively. After acetylation of α-amino

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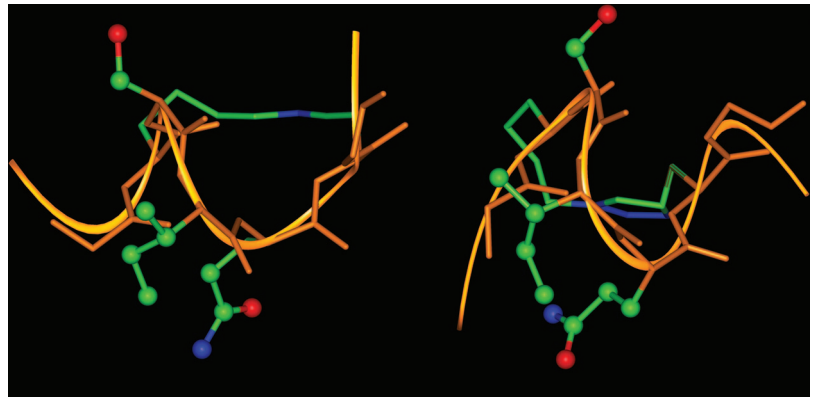

FIGURE 8. Comparison of representative NMR structures of lactam- and [1,2,3]triazolyl-containing cyclopeptides (left and right, respectively). Ser^{14}, Ile^{15}, and Gln^{16} side chains involved in the cycle are displayed in stick and ball presentation.

terminus with Ac_2O/NMM (2 mmol each, 114 and 189 μL, respectively) in a manner similar to any other N^{α}-Boc-amino acid, Fmoc, and Fm side-chain protecting groups were removed with 20% piperidine, the resin-bound peptide was washed with DMF and then treated for 2 h with 1.9 mmol of HBTU (3.8 mL × 0.5 M in DMF) and 1.9 mmol of DIEA to effect the lactam formation. The washed and dried resin-bound peptide was deprotected and cleaved with liquid HF in the presence of 5% *p*-cresol. The residue obtained after evaporating of the HF under vacuum was washed thoroughly with dry ether and treated with 0.5 M AcOH. The filtrate was lyophilized and the crude peptide lactam **II** was purified by HPLC-on a Vydac C_{18} prep column (5 × 30 cm, 15−20 μm) using a linear gradient 0−100% B in A at 40 mL/min over 2 h. Purity of >97% was confirmed by analytical HPLC (t_R = 6.76 min) using a Zorbax C_8 column (5 × 100 mm, 5 μm) and a linear gradient of 10−80% B in A at 1 mL/min over 15 min. The eluting solvent system was composed of A = 0.1% TFA in H_2O and B = 0.1% TFA in acetonitrile. The desired peptide lactam **II** was obtained in >98% purity; MS^+(MALDI-TOF) *m*/*z* 1067.90 ($[M + H]^+$), calcd 1066.6 (M^+).

Solid-Phase Synthesis of Ac[Nle^{13}(ε-N_3),Pra^{17}]hPTHrP(11−19)-NH_2. A. Via on-Resin Lys-to-Nle(ε-N_3) Transformation: I. Synthesis of Resin-Bound Ac[Lys^{11}(N^{ϵ}-Boc),Lys^{13}(N^{ϵ}-Mtt),Ser^{14}(tBu),Gln^{16}-(Trt),Pra^{17},Arg^{19}(N^{G}-Pbf)]hPTHrP(11−19)-Rink Amide Resin (1). The fully protected resin-bound peptide was synthesized using a modified ABI 430A peptide synthesizer. Hardware and software modifications employed follow the general scheme reported by Schnolzer et al.[55] except that Fmoc chemistry was used throughout. The peptide was synthesized on a Fmoc-Arg(N^{γ}-Pbf)-Rink amide MBHA resin on a 0.2 mmol scale. Deprotection of the temporary Fmoc group was achieved by 2 × 20 min treatments of the resin-bound peptide with 20% (v/v) piperidine in DMF. After extensive flow washing with DMF, coupling of each successive amino acid was achieved with 1 × 30 min incubation with the appropriate preactivated N^{α}-Fmoc-amino acid derivative. All protected amino acids (1 mmol) were dissolved in the cartridge with 3.8 mL of 0.25 M DEPBT in DMF as part of the synthesizer program immediately before delivery to the reaction vessel. Subsequently, 1 mL of DIEA was added directly to the cartridge to effect activation no more than two minutes prior to transfer of the coupling solution to the N^{α}-deprotected resin-bound peptide. After coupling was complete, the resin was extensively flow washed in preparation for the next deprotection/coupling cycle. Acetylation of the amino terminus was carried out in the presence of Ac_2O/NMM as coupling system described above.

The LC−MS analysis of a cleaved and deprotected sample obtained from an aliquote of the fully assembled resin-bound peptide revealed that the major peak in the chromatogram (t_R = 9.53 min; flow rate 1 mL/min; linear gradient 0−35% B in A in 20 min where A = 0.1% AcOH in water and B = 0.1% AcOH in acetonitrile) shows LC−ESI−MS $[M + H]^+$ = 1065.6 (calculated MW = 1064.6).

II. On-Resin Diazo-Transfer to Lys^{13}:

IIa. Selective Removal of Mtt Protecting Group from Lys^{13}. The resin-bound peptide **(1)** (0.31 mmol/g, 300 mg) was suspended in 1% TFA solution in DCM (10 mL) and was stirred for 3 min. The solution becomes yellow instantaneously. Then the resin was washed with DCM (2×), MeOH (1×), and DCM (2×). The process was repeated 8 times until the solution stayed colorless. The resin-bound peptide **1a** was taken to the next step without further manipulations.

IIb. On-Resin Transformation of Lys^{13} into Nle(ε-N_3).[34–36] Triflic acid anhydride (Tf_2O, 316 μL, 1.87 mmol) was added dropwise to a vigorously stirred mixture of NaN_3 (600 mg, 9.2 mmol) in H_2O (1.5 mL) and CH_2Cl_2 (3 mL) at 0 °C. The resulting mixture was allowed to warm to room temperature and was stirred for 2 h. The water layer was extracted twice with CH_2Cl_2, and the combined organic layers were washed with saturated aqueous Na_2CO_3. The resulting solution of TfN_3 in CH_2Cl_2 was added slowly to the resin-bound peptide **1a** suspended in a solution of $CuSO_4 \cdot 5H_2O$ (2 mg, 8 mmol) and K_2CO_3 (5 mg, 36 mmol) in MeOH (1 mL). This reaction mixture was swirled for 18 h at room temperature. The completeness of the diazo transfer could be followed with the Kaiser test; colorless resin beads implied that the conversion of the amino group into the azido functionality had been completed. The resin was subsequently washed sequentially with DCM, MeOH, DMF, and DCM.

III. Deprotection, Cleavage, and Purification of Free Peptide. The azide/alkynyl-containing resin-bound peptide was deprotected and cleaved from the solid support by treatment with TFA/TIS/H_2O (95:2.5:2.5 v/v) for 4 h at rt. After filtration of the resin, the TFA solution was concentrated under reduced pressure and precipitated in ether to yield the desired product as a solid. The corresponding product was identified by LC/MS with a X-Terra MS C_{18} column, 5 μm, 3 × 100 mm, flow rate 1 mL/min; t_R = 11.41 min using a linear gradient of 0 to 35% B in A in 20 min, where A = 0.1% AcOH in water and B = 0.1% AcOH in acetonitrile (crude: 58% purity at 214 nm).

The product (**2**) was purified by HPLC−MS, with a X-Terra Prep MS C_{18} column, 5 μm, 30 × 100 mm, using a linear gradient 0 to 35% B in A in 30 min, where A = 0.1% AcOH in water and B = 0.1% AcOH in acetonitrile with a flow rate = 20 mL/min. The desired product **2** was obtained in purity >98% (40 mg, yield = 39%). MS^+(ESI) *m*/*z* 1091.7 ($[M + H]^+$) calcd 1090.6 (M^+). Amino acid analysis (calcd/found): Glx 1/1.0; Ser 1/0.85;Gly 1/1.01; Arg 1/1.05; Ile 1/0.98; Leu 1/0.95; and Lys 1/1.01.

(56) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. *Anal. Biochem.* **1970**, *34*, 595–8.

B. Via Incorporation of Nle(ϵ-N_3): I. Synthesis of Resin-Bound Ac[Lys^{11}(N^{ϵ}-Boc),Nle^{13}(ϵ-N_3),Ser^{14}(tBu),Gln^{16}(Trt),Pra^{17},Arg^{19}(N^{G}-Pbf)]hPTHrP(11−19)-Rink Amide Resin (1). The fully protected resin-bound peptide was synthesized in manual synthesis following a procedure similar to the one described in section AI. The peptide was synthesized on a Rink amide resin (0.63 mmol/g) on a 0.19 mmol scale employing TBTU/HOBt/NMM (2.5 equiv/2.5 equiv/3.5 equiv) as the coupling system and 2.5 equiv of the Fmoc-protected amino acids except for Fmoc-Pra-OH (1.5 equiv) and Fmoc-Nle(ϵ-N_3)-OH (2 equiv) (see Supporting Information for synthetic details). Acetylation of the amino terminus was carried out in the presence of Ac_2O/NMM and was monitored by the Kaiser test.[56]

II. Deprotection, Cleavage, and Purification of Free Peptide. The azide/alkynyl-containing resin-bound peptide was deprotected and cleaved from the solid support by treatment with TFA/H_2O/EDT/phenol/anisole (94/2/2/2/2 v/v) for 3 h at room temperature. After filtration of the resin the TFA solution was concentrated under nitrogen flux and precipitated in cold diethyl ether to yield the desired product as a solid. The crude product (96% purity at 220 nm) was purified by preparative HPLC with a Jupiter C18 column, 10μm, 250 × 10 mm, flow rate 4 mL/min using a linear gradient of 10−60% B in A in 20 min, where A = 0.1% TFA in water and B = 0.1% TFA in acetonitrile. The desired product **2** was obtained as a white solid in purity >97% (18 mg, yield = 9%). The UPLC−MS analysis of a cleaved and deprotected sample obtained from an aliquot of the fully assembled resin-bound peptide revealed that the major peak in the chromatogram (t_R = 1.73 min; Flow rate 0.45 mL/min; linear gradient 10 to 60% B in A in 3 min where A = 0.1% TFA in water and B = 0.1% TFA in acetonitrile) shows LC−ESI−MS $[M + H]^+$ = 1091.7 (calculated MW = 1090.6).

[Ac-Lys-Gly-Xaa(&^{1})-Ser-Ile-Gln-Yaa(&^{2})-Leu-Arg-NH_2][(&^{1}$(CH_2)_4$-1,4-[1,2,3]triazolyl-CH_2&^{2})] (3). To a solution of a pure peptide (**2**) (10 mg, 9 μmol) in tBuOH/H_2O (1/2 v/v) (10 mL) were added $CuSO_4 \cdot 5H_2O$ (10 mg, 40 μmol) and ascorbic acid (10 mg, 60 μmol). The mixture was stirred ON at room temperature. Then, the solution was concentrated and lyophilized.

The corresponding product (**3**) was identified by LC/MS with a X-Terra MS C_{18} column, 5μm, 3 × 100 mm, using a linear gradient of 0 to 35% B in A in 20 min, where A is 0.1% AcOH in water and B is 0.1% AcOH in acetonitrile (crude: 82% purity at 214 nm), flow rate 1 mL/min; t_R = 9.04 min.

The product (**3**) was purified by HPLC−MS, with a X-Terra Prep MSC_{18} column, 5μm, 30 × 100 mm, using gradient eluting solvents: 0.1% AcOH in acetonitrile/0.1% AcOH in water (0−35% of acetonitrile in 30 min) to give the desired product (**3**) in high purity (95%) (5.5 mg, yield = 55%). MS^+(ESI) *m/z* 1091.7 ($[M + H]^+$), calcd 1090.6 (M^+).

Abbreviations Used

AMBER, assisted model building with energy refinement; Alloc, allyloxycarbonyl; DCM, dichloromethane; DEPBT, 3-(diethoxyphosphoryloxy)-3*H*-benzo[*d*][1,2,3]triazin-4-one; DIEA, *N*,*N*-diisopropylethylamine; DQF-COSY, double-quantum-filtered correlated spectroscopy; EDT, 1,2-ethaneditiol; ESI-MS, electron-spray ionization mass spectrometry; Fm, 9-fluorenylmethyl; GnRH, gonadotrophin-releasing hormone; HFA, hexafluoroacetone; HOBT, *N*-hydroxybenzotriazole; LC−ESI−MS, liquid chromatography−electron spray ionization−mass sprectrometry; Mtt, 4-methyltrityl; NMN, *N*-methylmorpholine; ON, overnight; Pac, phenacyl; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-ylsulfonyl; PDB, program database; Pra, propargylglycine; PTH, parathyroid hormone; PTHR1, PTH receptor 1; PTHrP, PTH-related peptide; RRE, Rev responsive element; rmsd, root-mean-square deviation; TBTU, 1-[bis(dimethylamino)methylene]-1*H*-benzotriazolium 3-oxide tetrafluoroborate; Tf, trifluoromethanesulfonyl; TFA, trifluoroacetic acid, TIS, triisopropylsilane; TOCSY, total correlated spectroscopy; Trt, trityl; UPLC, ultraperformance liquid chromatography.

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Supporting Information Available: Experimental procedure for the synthesis of Fmoc-Nle(ϵ-N_3)-OH, protocols for circular dichroism spectroscopy, NMR spectrometry, NMR structure calculation, molecular dynamics, and the corresponding references. This material is available free of charge via the Internet at http://pubs.acs.org.

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