Enzyme Inhibitors

DOI: 10.1002/ange.200805900

Probing the Bioactive Conformation of an Archetypal Natural Product HDAC Inhibitor with Conformationally Homogeneous Triazole-Modified Cyclic Tetrapeptides**

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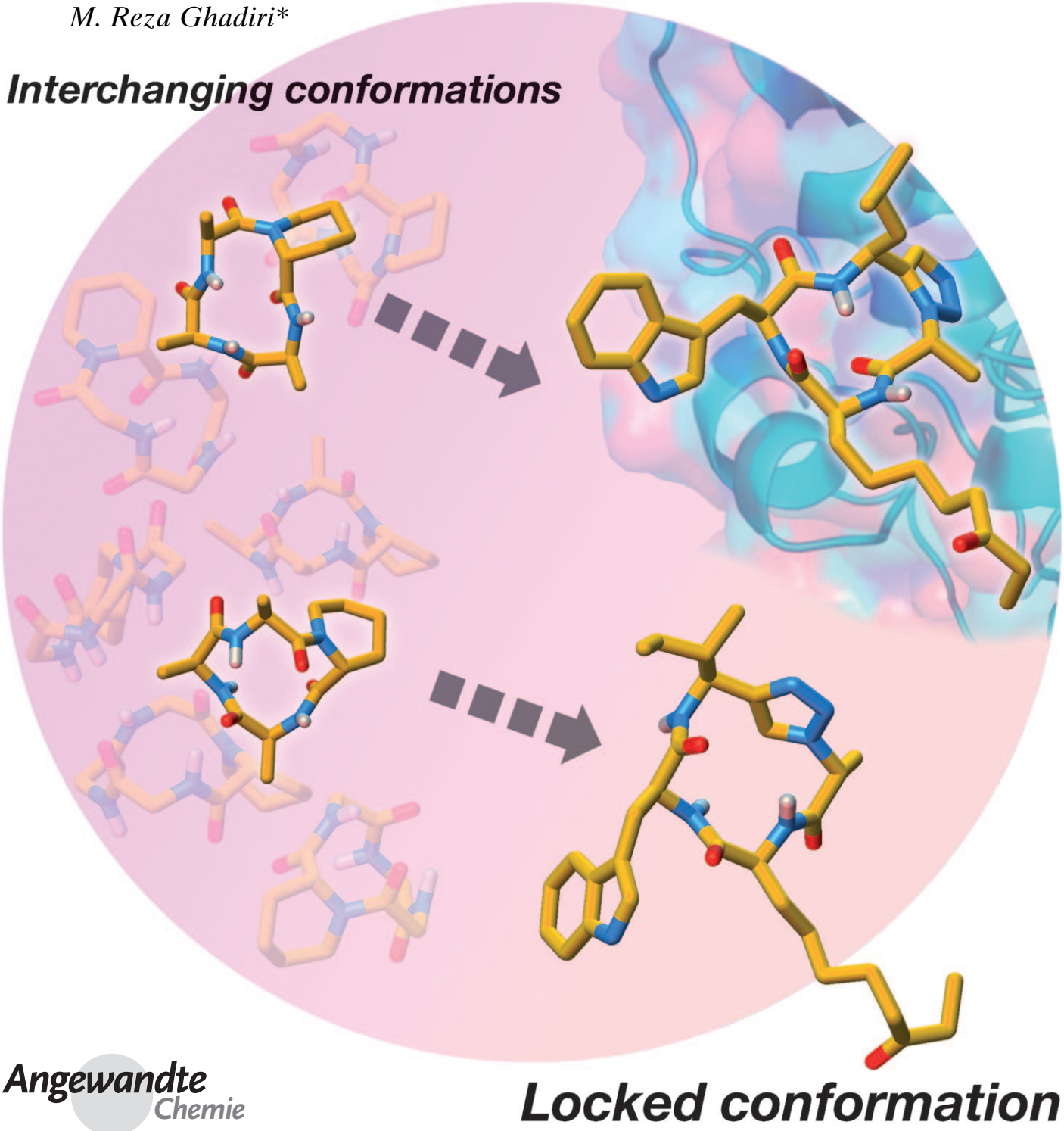

A fundamental strategy in the rational design of synthetic compounds to bind a protein of interest is to use a known ligand as a structural model to specify the precise conformational and pharmacophoric requirements for binding. Despite the remarkable success of this approach, a significant difficulty is that free ligands (in the absence of their cognate receptors) often adopt multiple conformations in solution or in the solid state.[1] These occurrences can render models designed on the basis of the free-ligand structure difficult to construct or even misleading.[2] Herein we present evidence that the more potent conformation of apicidin, an archetypal member of a family of naturally occurring cyclic tetrapeptide inhibitors of histone deacetylases (HDACs), is not the all-*trans* (*t-t-t-t*) structure that predominates in solution,[3,4] but rather a *cis–trans–trans–trans* (*c-t-t-t*) conformation. Our study involved the design, synthesis, structural characterization, and functional analysis of a series of cyclic pseudotetrapeptides containing 1,4- or 1,5-disubstituted 1,2,3-triazole amino acids, which serve as surrogates for *trans* and *cis* amide bonds, respectively. We show that by replacing an amide bond with a triazole, the bond in question can be fixed in either a *trans*-like or a *cis*-like configuration. In this way, we were able to probe the binding affinity of distinct conformations individually. The heterocyclic compounds adopt conformations that correspond closely to the targeted conformations of apicidin and demonstrate potent HDAC-inhibitory activities, in some cases equivalent or superior to those of the natural product. This study highlights the utility of triazole-modified cyclic peptides in the construction of useful bioactive probe molecules, supports the *c-t-t-t* conformation as the bioactive conformation of cyclic-tetrapeptide HDAC inhibitors, and provides a useful three-dimensional pharmacophoric model for use in advancing design principles for more selective HDAC inhibitors.

HDACs play a critical role in the regulation of gene transcription[5] by cleaving the acetyl groups from specific ε-aminoacetylated lysine residues in nucleosomal histone tails and nonhistone proteins.[6] Although the precise roles of HDAC isoforms in cellular function and tumorigenesis are not yet completely understood, the inhibition of HDAC activity has emerged as a promising approach in anticancer chemotherapy.[6–8] An interesting family of nonribosomal cyclic-tetrapeptide natural products, including the apicidins, trapoxins, microcins, and chlamydocin, exert potent cytotoxic activities against cancer cells by inhibiting HDACs.[6–9] These natural cyclic tetrapeptides are distinguished by the presence of a decanoic acid side chain derivatized with a ketone or terminal α,β-epoxyketone, which mimics an acetylated lysine residue and interacts with the active-site zinc ion.

Despite the considerable number of synthetic analogues that have been reported for the cyclic-tetrapeptide HDAC inhibitors,[8,10] the bioactive conformation has remained rather ambiguous. Indeed, several molecular conformations have been reported for apicidin alone (Figure 1). Apicidin was originally reported to adopt a *t-t-t-t* conformation on the basis of NMR spectroscopic experiments carried out with [D_5]pyridine or CD_2Cl_2.[4] More recently, our research group[11] and a group at Merck[3] found independently that apicidin adopts multiple conformations (in a ratio of approximately 80:15:5) in the more polar solvent DMSO, whereby the predominant species adopts a *t-t-t-t* conformation, and the

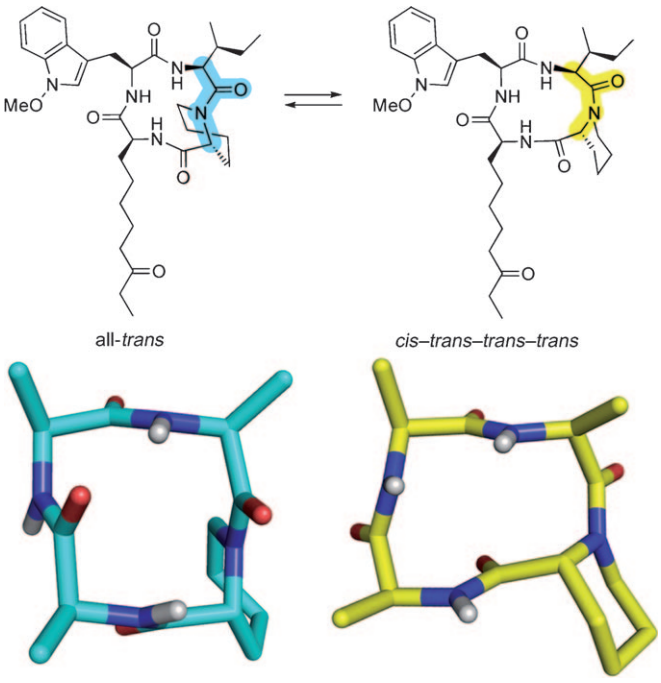

Figure 1. In dimethyl sulfoxide (DMSO), *cis–trans* isomerization in the cyclic-tetrapeptide natural product apicidin (**1**) gives rise to an equilibrium between the predominant *t-t-t-t* conformer (80%) and a minor *c-t-t-t* conformer (15%).[3,11] The molecular structures shown are the *t-t-t-t* structure determined by NMR spectroscopy in [D_5]pyridine[4] and the *c-t-t-t* crystal structure.[3]

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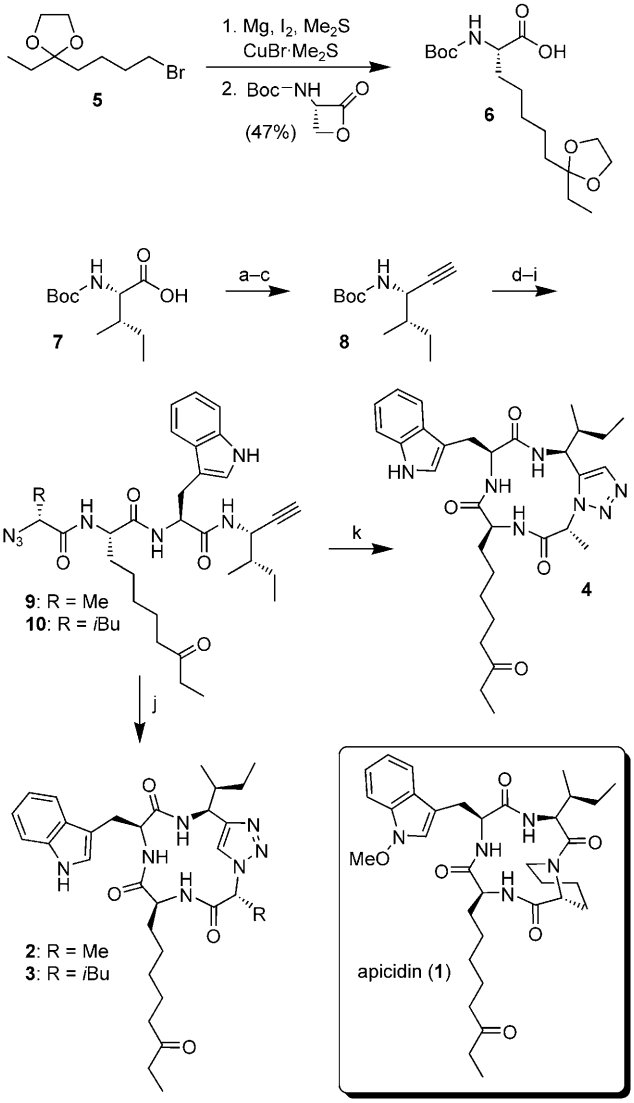
[**] We thank Dr. Dee Huang and Dr. Laura Pasternack for assistance with NMR spectroscopy, Dr. Sheo Singh for providing the coordinates of the published NMR structure of apicidin in [D_5]pyridine, Dr. Michael Kranz for providing the coordinates of the calculated lowest-energy conformations of apicidin, Dr. Michael Pique for assistance in the preparation of the frontispiece graphics, and Dr. L. J. Leman for assistance in manuscript preparation. We acknowledge the National Science Foundation (W.S.H.) and NASA Earth and Space Science Fellowship Program (grant NNX07AR35H; J.M.B.) for predoctoral fellowships. A.M. thanks the Spanish Ministry for Science and Education for a Fulbright/MEC postdoctoral fellowship. C.A.O. thanks the Lundbeck Foundation and the Danish Research Council for Technology and Production Sciences (274-06-0317) for postdoctoral fellowships, and the Danish Independent Research Council for a Young Researcher's Award. This research was supported in part by a grant from the National Institute of General Medical Sciences (GM52190) and by the Skaggs Institute for Chemical Biology. HDAC = histone deacetylase.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200805900.

tertiary amide bond of the pipecolic acid (Pip) residue in the second-most-populated conformation has a *cis* configuration. Finally, the crystallographic structure obtained for a crystal grown from a solution in $CHCl_3$/methanol/pentane showed the Pip residue to contain a *cis* amide.[3] The observation of both *cis* and *trans* tertiary amides in these structures of apicidin (as well as other tetrapeptide natural products[8,12]), combined with the lack of any cocrystal structure of a cyclic-tetrapeptide inhibitor with an HDAC enzyme, led us to question which amide configuration is present in the dominant bioactive conformation. Although the *c-t-t-t* conformation had previously been identified tentatively as the bioactive structure in related cyclic tetrapeptides on the basis of a correlation of activity with predicted or known solution conformations,[12] we aimed to carry out a more controlled study in which apicidin analogues with a fixed *cis*- or *trans*-amide isostere could be compared directly in an HDAC-inhibition assay.

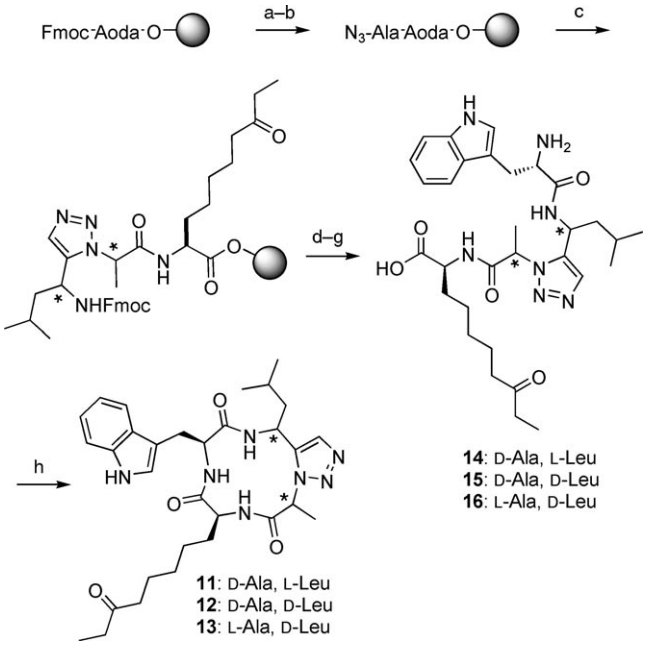
We and others have shown that the 1,4-disubstituted 1,2,3-triazole regioisomer effectively mimics a *trans* amide bond,[13] whereas the 1,5-disubstituted 1,2,3-triazole regioisomer is a good model for a *cis* amide bond.[14,15] Furthermore, the requisite 1,4- and 1,5-regioisomers can both be synthesized conveniently from azide and alkyne substrates.[16,17] Thus, the triazole moiety is an attractive choice for an amide-bond surrogate.

We first synthesized the heterocyclic apicidin analogues **2**, **3**, and **4** (Scheme 1). Bromoketal **5** was converted into the corresponding Grignard reagent and treated with Boc-protected serine β-lactone to yield the protected L-2-amino-8-oxodecanoic acid (L-Aoda) derivative **6**.[18] Boc-Ile (**7**) was converted into the propargylic amine derivative **8**,[19] which was used along with the amino acid **6** in solution-phase peptide synthesis to afford the linear azido–alkyne tetrapeptides **9** and **10**. A copper(I)-catalyzed intramolecular azide–alkyne cycloaddition[17] of **9** and **10** yielded the heterocyclic peptides **2** and **3**, respectively, each of which contains the *trans*-amide surrogate. Peptide **4**, with the 1,5-substituted 1,2,3-triazole, was prepared by a thermal Huisgen [3+2] dipolar cycloaddition of **9** (*N*,*N*-dimethylformamide (DMF), microwave, 220 °C), which afforded a mixture of the triazole regioisomers **2** and **4** in a 2:1 ratio. Peptide **4** was isolated from this mixture by preparative HPLC. The preparation of the additional 1,5-regioisomers **11**–**13**, in which the Ile residue was replaced with Leu, enabled us to investigate the hypothesis that the multiple conformations of **4** (see below) might arise from the presence of a β-substituted amino acid (Ile) adjacent to the triazole ring. Since the ruthenium-mediated cyclization of linear tetrapeptides with formation of the triazole moiety proved unsuccessful, we developed an alternative strategy, whereby the ruthenium-catalyzed formation of 1,5-disubstituted 1,2,3-triazoles on a solid phase was followed by macrolactamization of the linear pseudotetrapeptides **14**–**16** in solution to give **11**–**13** (Scheme 2; see also Figure S1 in the Supporting Information).[15,16] Compounds **11**–**13** differ only in the configuration at the Leu and Ala positions of the macrocycle; therefore, it was possible to examine how chirality might affect the conformational properties of the peptide ring. The Trp(OMe) residue present in apicidin was replaced with Trp in our compounds to simplify the syntheses.



Scheme 1. Synthesis of analogues **2**–**4** of apicidin (**1**): a) *N*,*O*-dimethylhydroxylamine·HCl, EDC·HCl, iPr$_2$EtN; b) $LiAlH_4$; c) dimethyl (2-oxopropyl)phosphonate, *p*-TsN_3, K_2CO_3 (48% over 3 steps); d) TFA; e) Boc-Trp-OH, HBTU, iPr$_2$EtN (94% over 2 steps); f) TFA; g) **6**, HBTU, iPr$_2$EtN (74% over 2 steps); h) TFA; i) N_3-D-Ala-OH or N_3-D-Leu-OH, EDC·HCl, HOBt, iPr$_2$EtN, 0 °C (73% for Ala, 31% for Leu over 2 steps); j) CuI, 2,6-lutidine, iPr$_2$EtN, tris(benzyltriazolylmethyl)amine[20] (50% (determined by HPLC)); k) microwave irradiation, 220 °C (**4**/**2** 2:1 (determined by HPLC), yield of isolated **4**: 8%). Boc = *tert*-butoxycarbonyl, EDC = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, HBTU = *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, HOBt = 1-hydroxy-1*H*-benzotriazole, TFA = trifluoroacetic acid, Ts = *p*-toluenesulfonyl.

Both 1,4-disubstituted regioisomers (**2** and **3**) showed a single set of sharp ^{1}H NMR peaks in [D_6]DMSO, which indicated their conformational homogeneity in solution.[21] Of the 1,5-disubstituted regioisomers, peptides **11** and **12** also adopted a single conformation on the NMR timescale, whereas analogues **4** and **13** showed multiple sets of



Scheme 2. Synthesis of analogues **11**–**13** with a 1,5-disubstituted triazole ring: a) NMP–piperidine (3:1); b) N_3-L-Ala-OH or N_3-D-Ala-OH (4 equiv), DIC (4 equiv), HOBt (4 equiv), NMP, 2 h; c) Fmoc-L-Leu-CCH or Fmoc-D-Leu-CCH (2 equiv), [Cp*Ru(cod)Cl] (20%), toluene, argon, 16 h, 45 °C; d) NMP–piperidine (3:1); e) Fmoc-Trp-OH (4 equiv), HBTU (4 equiv), *i*Pr_2EtN (8 equiv), NMP, 2 h; f) NMP–piperidine (3:1); g) TFA–CH_2Cl_2 (1:1); h) HATU (2 equiv), *i*Pr_2EtN (4 equiv), 1.5 h, 0.5 mM in DMF. All cyclization yields were greater than 95%, as determined by HPLC (see Figure S1 in the Supporting Information). Yields of the isolated products on the basis of resin loading were 9% for **11**, 9% for **12**, and 10% for **13**. cod = 1,5-cyclooctadiene, Cp* = pentamethylcyclopentadienyl, DIC = diisopropylcarbodiimide, DMF = *N*,*N*-dimethylformamide, Fmoc = 9-fluorenylmethoxycarbonyl, HATU = *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, NMP = 1-methyl-2-pyrrolidinone.

^{1}H NMR peaks indicative of multiple, slowly interconverting backbone conformations (see Figure S2 in the Supporting Information). The observed conformational heterogeneity of **4** and **13** possibly results from *cis–trans* isomerization of one or more amides in the macrocycles.

The bioactivities of the peptides were preliminarily assessed by using an in vitro fluorescence assay to measure the inhibition of HDAC activity in nuclear extracts of HeLa cells. Like apicidin, peptide **2** modified with the 1,4-disubstituted triazole and peptides **4**, **11**, and **12** modified with the 1,5-disubstituted triazole showed IC_{50} values of 100 nM or less. Compound **3** had an intermediate IC_{50} value of approximately 200 nM, whereas peptide **13** exhibited an IC_{50} value above 10 μM. As a control, the linear azido–alkyne peptide **9**, which contains all the important functional groups present in **3** and **4** but lacks the cyclic structure, had an IC_{50} value more than two orders of magnitude higher than that of any of the cyclic peptides. The lower inhibitory activity of **3** with respect to that of **2** may be explained by the methyl side chain in **2**, which may act as a better steric mimic of the Pip residue than the branched isobutyl side chain in **3**. Although compound **4** exhibited an IC_{50} value of less than 100 nM, the multiple conformations observed by ^{1}H NMR spectroscopy for this compound negated its utility as a probe for the bioactive conformation of apicidin. For the more detailed characterization of HDAC-inhibitory activities with a panel of purified recombinant human HDAC enzymes, we therefore employed only peptides **2**, **11**, and **12** (all of which adopted a single conformation, as indicated by ^{1}H NMR spectroscopy; Table 1). Against HDAC1, peptide **11** with a 1,5-disubstituted

Table 1: Observed conformations in DMSO and potency against recombinant human HDAC enzymes of triazole-containing pseudopeptides and control compounds.[a]

Compound	Conformation	IC_{50} [nM]			
		HDAC1	HDAC3[b]	HDAC6	HDAC8
2	*t-t-t-t*	25	16	>10000	ND[c]
11	*c-t-t-t*	7	9	6100	105
12	*c-t-c-t*	75	119	>10000	ND[c]
apicidin	*t-t-t-t* (80%) *c-t-t-t* (15%)	3	11	>10000	750
TSA[d]	–	ND[c]	ND[c]	6	32

[a] IC_{50} values were determined from the results of triplicate experiments. [b] HDAC3 was used in a complex with NCoR2 (nuclear receptor corepressor 2). [c] The IC_{50} value was not determined for the compound against this enzyme. [d] Trichostatin A (TSA) was used as a positive control for HDAC6 and HDAC8 because apicidin is a poor inhibitor of these enzymes.

1,2,3-triazole unit had an IC_{50} value similar to that of apicidin, whereas its regioisomer **2**, with the 1,4-disubstituted 1,2,3-triazole, showed an eightfold decrease in inhibitory activity relative to that of apicidin. Against HDAC3, the three compounds all had similar activities, although **11** was again somewhat more potent than **2**. Interestingly, compound **11** was significantly more active than apicidin against HDAC8, and somewhat more active against HDAC6. Compound **12**, which differs from apicidin in the configuration at the Leu residue, exhibited decreased activity relative to that of the other apicidin analogues for all HDACs tested.

The inhibition data obtained were somewhat surprising, as the greater activity of regioisomer **11** (with the 1,5-disubstituted 1,2,3-triazole) relative to that of **2** (with the 1,4-disubstituted 1,2,3-triazole) implicated the activity of the *c-t-t-t* conformation, even though the *t-t-t-t* conformation of apicidin predominates in solution in a variety of solvents. However, a critical requirement for the above analysis to be valid is that peptides **11** and **2** must closely mimic the targeted *c-t-t-t* and *t-t-t-t* conformations of apicidin, respectively. To confirm that the apicidin analogues adopted the intended conformations in solution, we carried out a series of structural analyses based on multidimensional NMR spectroscopy (TOCSY, COSY, and ROESY) and distance geometry calculations for compounds **2**, **11**, and **12** (all of which are conformationally homogeneous in [D_6]DMSO). The three-dimensional structure of **2**, as determined by NMR spectroscopy, indeed revealed that all backbone amide bonds were in the *trans* configuration (Figure 2a; see also Figure S3 in the Supporting Information). This conclusion was consistent with the observed large HN–H_α coupling constants (Trp 9.1 Hz, Ile 8.4 Hz, Aoda 8.6 Hz). An overlay of the NMR structure of **2**

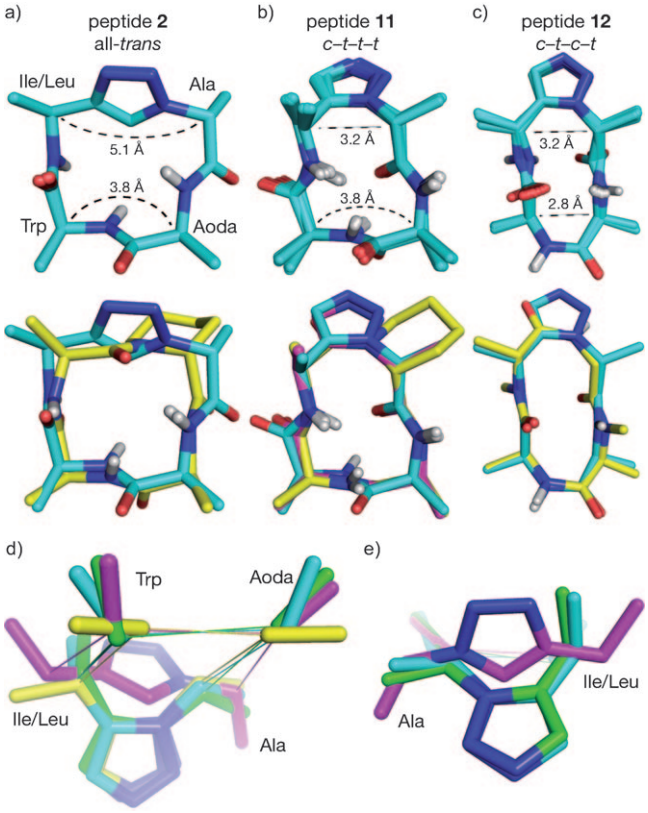


Figure 2. Structures determined by NMR spectroscopy for the triazole-modified apicidin analogues. a) Peptide **2** adopted a *t-t-t-t* conformation (top) that can be overlayed well (bottom; RMSD for C_α and C_β atoms: 0.52 Å) on the lowest-energy calculated conformation of apicidin (yellow), which reportedly matches closely the predominant conformation of apicidin in DMSO.[3] b) Peptide **11** adopted a *c-t-t-t* conformation; two families of structures were observed that differ in the rotation of the Trp/Aoda amide relative to the backbone (top). The structures can be overlayed very well with the crystal structure of apicidin[3] (bottom; RMSD for C_α and C_β atoms for representative members of the two structural families: 0.30 and 0.51 Å). c) Peptide **12** adopted a *c-t-c-t* conformation (top), which can be overlayed very well on the crystal structure of the natural product dihydrotentoxin[24] (yellow; bottom; RMSD for C_α and C_β atoms: 0.24 Å). d) Overlay of C_α atoms for compounds **2** (magenta), **11** (two structural families, green and cyan), and **12** (yellow). The *c-t-c-t* conformation of **12** causes the Aoda, Trp, and Leu side chains of this peptide to project in the same plane as the backbone ring, as opposed to projecting upward and out of the plane (as for the other peptides). e) Overlay of C_α and C_β atoms for compounds **2** (magenta) and **11** (two structural families, green and cyan). The C_α atoms of the Ala and Ile/Leu residues are farther apart in **2**, and the C_α–C_β vector of **2** directs the Ile side chain outward, away from the ring, as opposed to directly above the ring in **11**.

with the published *t-t-t-t* NMR structure of apicidin determined in [D_5]pyridine[4] indicated close similarity of the two structures with high backbone overlap and good C_α–C_β-vector alignments (backbone root mean square deviation (RMSD) from the mean structure for C_α and C_β atoms: 0.55 Å; see Figure S4 in the Supporting Information). Likewise, overlaying of the NMR structure of **2** with the lowest-energy calculated conformation of apicidin, which reportedly matches the conformation of the predominant species in [D_6]DMSO closely,[3] indicated a significant backbone overlap and good alignment of the C_α–C_β vectors (RMSD for C_α and C_β atoms: 0.52 Å; Figure 2a and Figure S3 in the Supporting Information). The reported NMR solution structures determined in [D_5]pyridine and [D_6]DMSO for apicidin differ only in the rotation of the Aoda–Trp amide bond relative to the plane of the backbone; the corresponding amide bond in compound **2** adopts the rotamer conformation present in the structure of apicidin determined in [D_5]pyridine.

Peptides **11** and **12**, which differ only in the stereochemistry at the Leu residue, adopt considerably different backbone conformations in [D_6]DMSO. The three amide bonds of peptide **11** (in which the amino acid residues have the same configuration as in apicidin) were all in the *trans* configuration. This arrangement corresponds to the *c-t-t-t* conformation if the 1,5-disubstituted 1,2,3-triazole is considered as a *cis*-amide-bond isostere (Figure 2b and Figure S3 in the Supporting Information). Two families of structures that differed only in the rotation of the Aoda–Trp amide bond relative to the backbone plane were observed for **11**; however, this amide rotation has little effect on the position of other backbone atoms or on C_α–C_β vectorial alignments (RMSD for C_α and C_β atoms of representative members of the two families: 0.28 Å). The structures determined by NMR spectroscopy for **11** could be overlayed very well on the crystallographic *c-t-t-t* structure of apicidin[3] (RMSD for C_α and C_β atoms of representative members of the two families: 0.30 Å and 0.51 Å; Figure 2b and Figure S3 in the Supporting Information). Thus, **11** indeed appears to offer a faithful representation of the *c-t-t-t* conformation of apicidin that is present as a minor component in solution in [D_6]DMSO. On the other hand, peptide **12** contained a *cis* configuration at the Aoda–Trp amide bond and therefore a *cis–trans–cis–trans* (*c-t-c-t*) tetrapeptide conformation (Figure 2c and Figure S3 in the Supporting Information). The *c-t-c-t* structure has been the focus of several calculations and discussions in the literature,[12,22,23] probably because it is among the most common conformations observed in the structures of cyclic tetrapeptides and is reportedly the conformation with the least ring strain. Although our data indicate that the *c-t-c-t* conformation is poorly suited to exert potent HDAC-inhibitory activity, peptide **12** could be useful as a lead compound in the construction of conformationally restricted analogues of other cyclic-tetrapeptide natural products that are known to adopt the *c-t-c-t* conformation, such as tentoxin,[23] dihydrotentoxin,[24] and the symmetric natural products cyclo(L-Pro-L-Leu)$_2$, cyclo(L-Pro-L-Val)$_2$, and cyclo(L-Pro-L-Phe)$_2$.[25] Indeed, peptide **12** could be overlayed closely on the crystal structure of dihydrotentoxin[24] (RMSD for C_α and C_β atoms: 0.24 Å; Figure 2c and Figure S3 in the Supporting Information).

Although there are subtle variations in the chemical structures of the natural product **1** and analogues **2**, **11**, and **12**, it is our hypothesis that the respective *cis*/*trans* configurations and resulting backbone conformations in each cyclic peptide are the major determinant of the observed HDAC-inhibition potencies.[26] For example, peptide **12** exhibited decreased HDAC-inhibitory activity relative to that of **2** and **11** across our panel of HDAC enzymes. An overlay of

C_α atoms for the three compounds clearly shows that compound **12** differs structurally from the other two peptides: The distance between the Trp and Aoda C_α atoms is significantly shorter in **12** (2.8 Å for **12**; 3.8 Å for both **2** and **11**), and, perhaps more importantly, the chairlike *c-t-c-t* conformation of **12** causes the C_α–C_β vectors for the Trp, Aoda, and Leu side chains to all project in the same plane as the backbone ring, whereas these side chains in **2** and **11** project above the plane of the ring (Figure 2d). Peptides **2** and **11** exhibited similar IC_{50} values against HDAC3, whereas **11** was four times as potent as **2** against HDAC1. An overlay of C_α and C_β atoms indicated that the most obvious structural differences between the two compounds are the greater distance between C_α atoms of the Ile/Leu and Ala residues in **2** (5.1 Å for **2**; 3.2 Å for both structural families of **11**) and the different directions in which the Leu or Ile residue is projected relative to the backbone ring (directly above the ring in **11** and outward from the ring in **2**; Figure 2e). Together with the biological data, this finding suggests that the position and orientation of the Leu/Ile residue influence potency against HDAC1, but do not have a pronounced effect on potency against HDAC3. It is also possible that the difference in the aliphatic residue in **2** and **11** (Ile in **2** and Leu in **11**) contributes to the observed differences in activity for these compounds.

In summary, the structural and functional data presented herein suggest that a *cis* configuration at the Pip residue in the cyclic-tetrapeptide HDAC inhibitor apicidin affords improved HDAC-inhibitory activities over those of the *t-t-t-t* conformation that predominates in solution. We have described the rational design of conformationally constrained, triazole-modified cyclic-peptide scaffolds with potent biological activity and have established the ability to probe the biologically relevant conformation of a natural peptide ligand by introducing different triazole regioisomers in place of amide bonds in its backbone. It is our hope that this study will help guide future efforts aimed at improving on the cyclic-tetrapeptide HDAC inhibitors and lead to more selective HDAC ligands.

Coordinates for the NMR structures reported in this manuscript have been deposited at the BMRB databank (www.bmrb.wisc.edu, accession numbers 20071–20073).

Received: December 4, 2008
Published online: March 6, 2009

Keywords: cyclic peptides · drug design · enzyme inhibitors · structure–activity relationships · triazoles

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