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Methylation-mediated control of aurora kinase B and Haspin with epigenetically modified histone H3 N-terminal peptides

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ABSTRACT

If multiple post-translational modifications are responsible for important biological markers, additional specificity must be present to serve as embedded combinatorial markers for phosphorylation. In this investigation, we have attempted to elucidate the specificity of AURKB and Haspin by using peptides of various lengths that contain all possible methylations, acetylations, and phosphorylations in histone H3 N-terminal peptides. The activity of AURKB is affected by a wide range of modifications from R2 to K14, while that of Haspin is affected significantly by modifications at R2 and K4. In cases where kinase activity is reduced substantially by other modifications, dimethylation at R2 and R8 totally abolishes phosphorylation at S10 promoted by AURKB and as does dimethylation at R2 on Haspin promoted phosphorylation at T3.

1. Introduction

Epigenetic changes brought about by post-translational modifications (PTM) are widely used to generate on/off switches for protein–protein interactions, which serve as important gene controls in cells.[1] Phosphorylation, methylation, and acetylation are the three most frequent chemical processes involved in PTMs. In addition, two or more PTM events can be employed simultaneously in order to modulate signals.[2] N-Terminal histone proteins have extensive epigenetically varied sequences, especially in H3,[3] that are decorated by different types of PTMs (Fig. 1). In order to accomplish multiple modifications, one change should be carried out in the presence of other PTMs and, as a result, the possibility exists that one PTM could influence the nature and efficiency of a subsequent PTM. Since they are accompanied by relatively large structural and environmental changes and they are easily recognized, phosphorylation reactions are often used as initial signals to promote other PTMs. Indeed, phosphorylation-mediated signaling is known to affect methylation or acetylation based processes in H3.[4] For example, phosphorylation at S10 results in suppression of mono- and dimethylation at K9[5] and an enhancement of acetylation at K14.[6] The results of a recent study also showed that a variety of methylations, taking place in the presence of phosphorylation mimicry of S10, are retarded significantly.[7]

However, few examples exist where phosphorylation processes are affected by other PTMs. In terms of molecular recognition, methylation reactions do not bring about as dramatic changes in structure or environment as do phosphorylation processes. Furthermore, the different sites as well as multiple patterns of methylation that occur in Lys (mono-, di-, and tri-) and Arg (symmetric and asymmetric di-), which are components of 'histone codes,'[8] complicate investigation of the roles of methylation as signal events. Single methylation/demethylation processes are not easily detected since generation of antibodies against individual, complex methylation patterns are difficult if not impossible. Also, the old belief that methylations are static changes[9] has hampered investigations of methylation-mediated signalling.

Aurora kinase B (AURKB) and haploid cell-specific protein kinase (Haspin) are two well-known participants in N-terminal histone H3 phosphorylations during mitosis.[10] A related role played by these kinases relates to the fact that AURKB is activated by Haspin promoted phosphorylation at T3.[11] Irrespective of the presence of several other sites, AURKB promotes phosphorylation at S10 and S28 solely. This finding indicates that amino acid sequences before and after the phosphorylation site must govern phosphorylation specificity.[12] A conserved RKSP amino acid sequence at the phosphorylation site exists not only in the N-terminal histone H3 but also in the RNA methyltransferase NSUN2 of vertebrates.[13a] T3 residue of N-terminal histone H3 is the only known phosphorylation site for Haspin as its substrate. Phosphorylation of H3 N-terminal is directly recognized by Survvin, which is a component of chromosomal passenger complex during mitosis.[13b]

Even though phosphorylation is one of the most important PTMs in histone H3, previous observations suggest that this process is not the sole type of modification that leads to physiological

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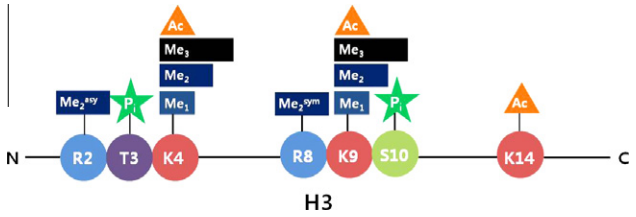

Figure 1. Post translational modifications found at the end of N-terminal histone H3.

changes. For example, there are novel histone H3 methyltransferases that are required for heterochromation formation.[13c] If multiple PTMs are responsible for important biological marking, additional specificity must be present to serve as embedded combinatorial markers for phosphorylation.[14] A brief comment was made describing the control of Haspin phosphorylation rates by neighbouring methylations,[15] However, a comprehensive study of the variations of AURKB and Haspin activities by epigenetic PTMs of neighboring amino acids has not been conducted yet despite the fact that cooperative interactions exist for this phosphorylation process during histone mediated cellular events.[16,17] In the current investigation, we have attempted to elucidate the specificities of AURKB and Haspin by using peptides that contain all possible PTMs in the histone H3 N-terminal peptides. Kinase activities of AURKB and Haspin are reduced in a manner that depends on the position and type of the PTM. In cases where the kinase activity is reduced substantially by other modifications, dimethylation at R2 and R8 leads to totally abolishment of phosphorylation at S10 by AURKB and dimethylation at R2 and trimethylation at K4 causes the same effect on phosphorylation at T3 by Haspin.

2. Results and discussion

2.1. Construction and usage of histone H3 N-terminal fragment peptides

At the beginning of this effort, the amino acid sequence requirements for the proper activity of AURKB and Haspin were elucidated using the sequence of the histone H3 N-terminal peptide. Peptide sequences of variable amino acid (aa) lengths, including those at the N-terminals were synthesized (Table 1) using solid-phase procedures.[18] The longest peptide generated, containing 18 aa residues, is identical with the histone H3 N-terminal peptide.[19]

Each of the prepared peptides was subjected to kinase catalyzed phosphorylation using high substrate concentrations (20 μM). The results show that AURKB has a length-dependent specificity. Peptide 17 (Table 1), corresponding to the 1–12 N-terminal residues of H3, displays a compatible kinase activity (75%) relative to the 18 aa peptide, whereas peptides with less than 10 aa moieties show negligible activities. Peptide 11, corresponding to the 2-11 N-terminal residues, has only 42% of the activity of peptide 23. Haspin displays almost full activity (90%) with 10 aa long peptides (Peptide 10) relative to the 18 aa peptide. The results show that two amino acids prior to T3 are essential for Haspin activity, since elimination of the amino acids prior to T3 leads to negligible kinase activity (Peptide 7, 12, 15, 18, and 20 in Table 1).

2.2. Construction of epigenetically modified histone H3 peptides and determination of kinetic constants (k_{cat} and K_M)

Libraries of peptides, corresponding to epigenetically modified N-terminal histone H3 peptides, were prepared next (Table 2). Two peptide libraries, based on peptide 23 (18 aa) and peptide 17 (12 aa), were targeted in this effort. Despite the fact that peptide

Table 1
Fragment of H3 N-terminal peptides used as substrates for AURKB and Haspin promoted phosphorylations and their relative activities

Peptide	Number of aa	Sequence	AURKB Activity[a]	Haspin Activity[b]
1	4	ARTK————————	—	21
2	4	————ARKS———————	0	—
3	6	ARTKQT————————	—	40
4	6	——QTARKS———————	0	—
5	6	————ARKSTG————	0	—
6	8	ARTKQTAR—————————	—	68
7	8	–TKQTARKS———————	2	0
8	8	——QTARKSTG————	0	—
9	8	————ARKSTGGK——	23	—
10	10	ARTKQTARKS———————	28	90
11	10	-RTKQTARKST———————	42	31
12	10	–TKQTARKSTG————	13	4
13	10	——QTARKSTGGK——	13	—
14	10	————ARKSTGGKAP–	16	—
15	11	-RTKQTARKSTG————	62	15
16	11	—KQTARKSTGGK——	13	—
17	12	ARTKQTARKSTG————	75	100
18	12	–TKQTARKSTGGK——	43	0
19	13	ARTKQTARKSTGG———	87	100
20	13	-RTKQTARKSTGGK——	38	0
21	14	ARTKQTARKSTGGK——	78	100
22	16	ARTKQTARKSTGGKAP–	58	100
23	18	ARTKQTARKSTGGKAPRK	100	100

[a] AURKB (1.5 nM) at 30 °C for 30 min, 50 μM ^{32}P-ATP, 20 μM of substrate.
[b] Haspin (1.5 nM) at 30 °C for 30 min, 750 μM ^{32}P-ATP, 20 μM of substrate.[20] Activity is % of phosphorylation relative to that of 18 aa peptide 23.

17 (12 aa) has 75% of the activity of peptide 23 in AURKB promoted phosphorylation, variations of this peptide were prepared owing to their ease of synthesis. Furthermore, peptide 17 and its derivatives are ideal short substrates since they contain phosphorylation sites at T3 and S10, all of the possible mono-, di-, and tri-methylation or acetylation sites in K4 and K9,[21] and symmetric and asymmetric dimethylation sites in R2 and R8.[22] Libraries of mono-site variable peptides containing 12 aa and 18 aa were constructed by inserting one modified Lys or Arg residue in place of the normal Lys or Arg residues (Table 2). The AURKB and Haspin activities of the epigenetically modified peptides were then evaluated by determining k_{cat} and K_M values (Figure ure2). The differences in the k_{cat}/K_M values mainly derive from variations in the K_M rather than k_{cat} values

Table 2
Mass spectrometric data[a] of epigenetically modified peptides[b]

Modification	12 aa		18 aa	
	Calcd.	Obsd.	Calcd.	Obsd.
None	1303.75	1303.86	2166.23	2167.54
R2Me$_2^{sym}$	1331.78	1331.83	—	—
R2Me$_2^{asy}$	1372.79	1373.89	—	—
T3Pi	—	—	2062.16	2062.72
K4Me	1358.77	1359.91	2180.24	2180.42
K4Me$_2$	1372.79	1374.05	2194.26	2196.62
K4Me$_3$	1387.81	1387.83	2251.29	2252.58
K4Ac	1386.77	1387.52	2208.24	2210.38
R8Me$_2^{sym}$	1372.79	1374.00	—	—
R8Me$_2^{asy}$	1331.78	1331.02	—	—
K9Me	1358.77	1359.90	2180.24	2182.35
K9Me$_2$	1372.79	1374.06	2194.26	2194.26
K9Me$_3$	1387.81	1387.06	2209.28	2210.63
K9Ac	1386.77	1388.11	2208.24	2210.05
S10Pi	—	—	2062.16	2062.70
K14Ac	—	—	2250.20	2251.60
T3PiS10Pi	—	—	2142.16	2142.87

[a] Mass spectrometric data for the peptides represents $[M+H]^+$.
[b] Each number and abbreviation represents PTM at the designated position. Peptides with 18 aa were biotinylated for various applications.

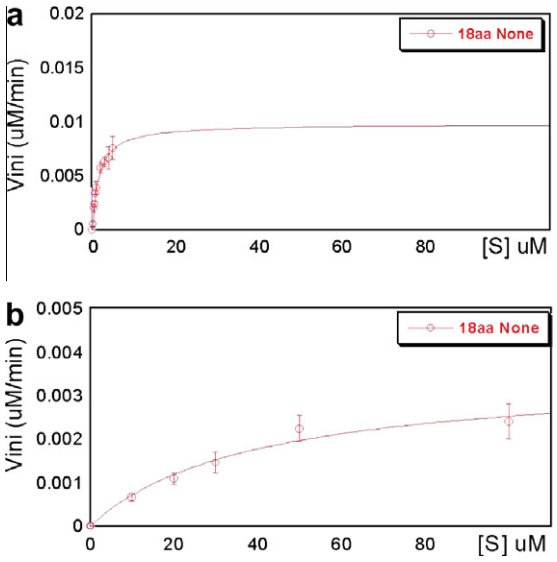

Figure 2. Michaelis-Menten analysis of phosphorylation kinetics of peptide 23 (18 aa) by (a) AURKB and (b) Haspin to determine K_M and k_{cat}. All data points were triplicated and averaged.

for AURKB, while k_{cat}/K_M differences for Haspin are a consequence of variations in both k_{cat} and K_M. These data, along with the peptide length dependent activities describe above, suggest that AURKB possesses a wide substrate range, and the substrate range for Haspin is narrow (Table 3). The activities of both kinases on peptide substrates containing other modifications were either slightly or significantly lower (>28-fold) except in a few cases where the activities are higher.

2.3. Effects of histone H3 modifications on AURKB and Haspin activities

Most peptides containing methyl modifications at both K4 and K9 have reduced activities for AURKB, except for K4Me (Table 3), which displays a 1.3-fold increase in activity relative to that of the unmodified peptide. The most greatly reduced kinase activity is seen with $K9Me_2$ (sixfold). This is an interesting result since recent data obtained from total proteome analysis reveal that the major variant of K4 is mono-methylation while the major variant of K9 is dimethylation.[23,24] The peptide with the second most reduced activity is $K4Me_3$ (threefold). This methylation modification ($K4Me_3$) causes a significantly lower Haspin activity (>28-fold), other whereas methylations at K4 and K9 have little effect on the activity of this kinase. This finding suggests that only modifications close to T3 alter the activity of Haspin. In contrast, phosphorylation by AURKB is controlled by methylations at neighboring as well as remote sites.

The peptide K4Ac, modified by acetylation, was found to have respective sevenfold and 23-fold reduced activities with AURKB and Haspin in comparison with those of the unmodified substrate. While the K9Ac modified peptide had a slightly lower activity with AURKB (twofold) and Haspin (twofold), its K14Ac analog displayed markedly lower activities against AURKB (sixfold) and Haspin (ninefold). Overall, the large reductions of activities caused by acetylation are greater than those induced by all methylation modified peptides (except that seen in $K4Me_3$ for Haspin) suggesting that the presence of amine rather than amide moieties at K4 and K14 is important for recognition by both kinases.

The effect of Arg methylation was explored by using 12 aa peptides.[25] Surprisingly and importantly, the worst AURKB substrates among those probed are $R2Me_2^{asy}$ (14-fold) and $R8Me_2^{sym}$ (not calculable, >28-fold, Table 3). Moreover, Haspin activity is totally absent in $R2Me_2^{asy}$ (>28-fold), but not in $R8Me_2^{sym}$. We were curious about the effects of dimethylation on Arg, even though this type of modification profile is not seen in nature per se. As a result, two modified peptides, $R2Me_2^{sym}$ and $R8Me_2^{asy}$, were synthesized and tested as substrates for both kinases. AURKB was found not to act upon $R8Me_2^{asy}$ (>28-fold) but $R2Me_2^{sym}$ did serve as substrate for this kinase (Table S3). In addition, Haspin activity was absent for both $R2Me_2^{sym}$ (28-fold) and $R8Me_2^{asy}$ (>28-fold, Table S4). The data suggest that both symmetric and asymmetric dimethylation modifications at proximal Arg residues (R8 for AURKB and R2 for Haspin) hinders important interactions, such as hydrogen bonding, between the methylated substrates and amino acid residues in the active sites of the kinases, and that this effect leads to complete loss of activity at S10 and T3. Since dimethylation at R8 leads to a larger AURKB activity reduction than any other modifications close to K9, this modification might be the most affective in governing the kinase activity of the enzyme. A recent report[7] that insertion of Glu to mimic phosphorylated S10 retards methylation at R8 and that no dual modifications are found at both R8 and S10

Table 3
Kinetic constants of modified 18 aa peptides toward AURKB[a] and Haspin[b]

Modification	AURKB			Haspin		
	k_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M ($\mu M^{-1}min^{-1}$)	k_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M ($\mu M^{-1}min^{-1}$)
None	11	1.1	10	8.4	46	0.18
K4Me	12 (1.1)	0.94 (1.2)	13 (1.3)	8.8 (1.0)	41 (1.1)	0.21 (1.2)
$K4Me_2$	11 (1.0)	1.3 (1.2)	8.5 (1.2)	8.3 (1.0)	120 (2.6)	0.069 (2.6)
$K4Me_3$	7.8 (1.4)	2.5 (2.3)	3.1 (3.2)	NC	NC	NC (>28)
K9Me	11 (1.0)	1.5 (1.4)	7.3 (1.4)	10 (1.2)	31 (1.5)	0.32(1.8)
$K9Me_2$	10 (1.1)	5.9 (5.4)	1.7 (5.9)	9.7(1.2)	42 (1.1)	0.23(1.3)
$K9Me_3$	8.7 (1.3)	2.0 (1.8)	4.4 (2.3)	8.1(1.0)	52 (1.1)	0.16 (1.1)
K4Ac	9.7 (1.1)	6.9 (6.3)	1.4 (7.1)	0.44 (19)	55 (1.2)	0.0080 (23)
K9Ac	8.3 (1.3)	1.4 (1.3)	5.9 (1.7)	7.6 (1.1)	99 (2.2)	0.077 (2.3)
K14Ac	5.3 (2.1)	3.3 (3.0)	1.6 (6.3)	1.6 (5.3)	77 (1.8)	0.021 (8.6)
T3Pi	2.1 (5.2)	2.7 (2.5)	0.78 (13)	–	–	–
S10Pi	–	–	–	4.6 (1.8)	270 (5.9)	0.017 (11)
T3PiS10Pi	–	–	–	–	–	–
[c]$R2Me_2^{asy}$	4.2 (2.6)	5.8 (5.3)	0.72 (14)	NC	NC	NC (>28)
[c]$R8Me_2^{sym}$	NC	NC	NC (>28)	8.2 (1.0)	60 (1.3)	0.14(1.3)

[a] Conditions: 1.5 nM AURKB at 30 °C for 10, 20, and 30 min; 50 μM ^{32}P-ATP.
[b] Conditions: 1.5 nM Haspin at 30 °C for 10, 20, and 30 min; 750 μM ^{32}P-ATP. All data are averages from at least triplicate experiments. Numbers in parenthesis are fold-increase (red) and -decrease (blue) of activities of each peptide relative to unmodified one.
[c] Peptides with 12 aa were used for determining kinetic constants of Arg methylation due to the ease of synthesis.

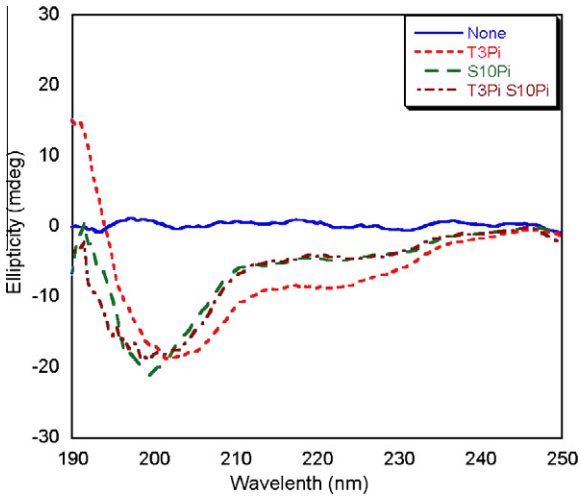

Figure 3. CD spectra of none modified and phosphorylated 18 aa peptides.

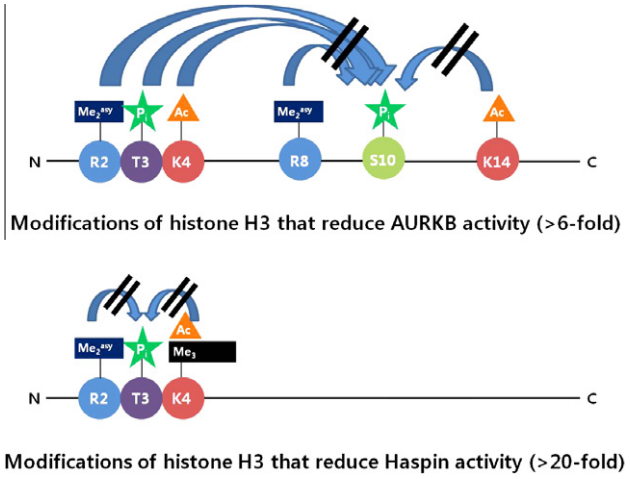

Figure 4. Summary of modifications at H3 histone that reduce activities of AURKB and Haspin significantly.

suggests that methylation at R8 and phosphorylation at S10 by AURKB might act as a reverse signal in mitosis. Even though initial trials have met with failure, continuing studies are underway to gain biological evidences for this banning signal.[26]

Phosphorylations promoted by AURKB and Haspin at S10 (eight-fold) and T3 (11-fold) were significantly retarded by pre-existing phosphorylations at T3 and S10, respectively (Table 3). These results suggest that dual phosphorylation at T3 (i) and S10 (i+7) positions does not likely occur over short distances. However, another phosphorylation site for AURKB exists at S28,[27] where no neighboring dimethylation at R26 and no preference for methylation at K27 upon phosphorylation were found during mitosis.[28] CD spectroscopic analysis showed that the T3 phosphorylated peptide displays an apparent increase in α-helical content (Fig. 3), owing to the potential existence of salt bridges between phos phorylated T3 and close basic side chains. In contrast, the dual T3 and S10 phosphorylated peptide did not have a modified secondary structure.[29] These observations once again provide support for the proposal that dual phosphorylations at both T3 and S10 are not likely. It would be interesting to investigate another kinase, VRK1, which phosphorylates both T3 and S10 of histone H3 during mitosis in order to determine if dual phosphorylation is a critical 'histone code'.[30]

3. Conclusion

In summary, a variety of epigenetically modified short peptides that mimic the H3 histone N-terminus were prepared and their activities as substrates for AURKB and Haspin promoted phosphorylation were determined in this effort. The data obtained in this effort demonstrate that the activity of AURKB is sensitive to short- and long-range modifications, while that of Haspin is sensitive to only short-range modifications. The most important findings in this work are that (1) dimethylation at R2 and R8 leads to peptides that have non-existent activities for AUKRB and Haspin, (2) peptides with methylation modifications at K4 and K9 have reduced rates of phosphorylation promoted by AURKB and Haspin, while dimethylation as seen in K4Me$_3$ has a significant effect in diminishing the activity of Haspin, (3) phosphorylation at T3 or S10 significantly reduces the rates of phosphorylation at the other site suggesting that a dual phosphorylation at T3 and S10 might not be one of as 'histone code', and (4) peptides containing acetylation at K4 and K14 have greatly reduced kinase activities for both AURKB and Haspin.[31] As shown in Figure 4, AURKB is affected by a wide range of modifications close to or distant from R2 to K14, while Haspin is only significantly affected by modifications at R2 and K4. In vitro kinetic studies, using epigenetically modified short N-terminal peptides mimicking histone H3, could provide important information leading to a greater understanding of the relationship between histone H3 N-terminal modification patterns and in vivo kinase activities.

4. Experimental

4.1. Synthesis of peptides

A modified library of histone H3 N-terminal peptides was synthesized by using the standard Fmoc solid-phase peptide synthesis protocol.[32]

4.2. AURKB assay

Recombinant human AURKB was purchased from SignalChem and kinetic assay using AURKB was carried out using a minor modification of the manufacturer's protocol. Briefly, various concentrations of peptides were incubated with 2.5 ng/reaction (1.5 nM) of aurora B, 50 μM of ^{32}P-ATP assay cocktail in 5 mM MOPS, pH 7.2, 2.5 mM β-glycerol-phosphate, 5 mM $MgCl_2$, 1 mM EGTA, 0.4 mM EDTA, 0.05 mM DTT for 10, 20, and 30 min at 30 °C. Reactions were terminated by spotting samples onto individual pre-cut strips of P81 phophocellulose paper (Upstate). The dried papers were washed three times with 1% phosphoric acid and then mixed with scintillation fluid (Perkin Elmer) and analyzed by using a liquid scintillation counter (Wallac 1409 DSA). K_M and k_{cat} values were calculated by using the Michaelis–Menten equation.

4.3. Haspin assay

Recombinant active Haspin was purchased from Millipore. Various concentrations of peptides were incubated with 1.5 ng/reaction (1.5 nM) of Haspin, 750 μM of ^{32}P-ATP assay cocktail in 50 mM HEPES at pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 150 mM NaCl, 2 mM DTT for 10, 20, and 30 min at 30 °C. After incubation, reactions were terminated by spotting of samples onto individual pre-cut strips of P81 phophocellulose paper (Upstate). Dried papers were washed three times with 1% phosphoric acid and then counted radioactivity in the presence of 2 mL scintillation fluid (Perkin Elmer) in a liquid scintillation counter (Wallac 1409

DSA). K_M and k_{cat} values were calculated by Michaelis–Menten equation.

4.4. CD study

CD measurements were performed at 20 °C on a JASCO model J715 spectropolarimeter equipped with Peltier temperature controller and running JASCO Spectra Manager™ software. 100 μM peptides in a buffer solution containing 10 mM H_3PO_4 at pH 7.4 with 50% TFE at 20 °C.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.02.011.

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