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Graphical Abstract

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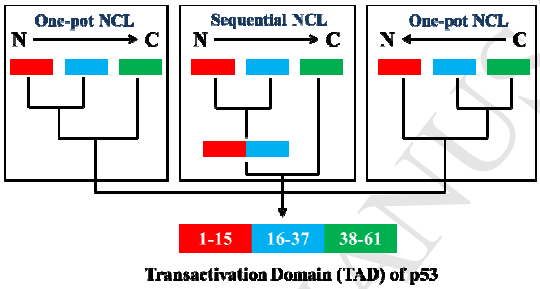

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ABSTRACT

Chemical composition of tumor suppressor protein p53 is altered via multiple post-translational modifications which modulate its cellular lifetime and interactions with other biomolecules. Here we report total chemical synthesis of a 61-residue form of transactivation domain (TAD) of p53 based on native chemical ligation of three peptide segments. The experiments to characterize its binding to nuclear co-activator binding domain (NCBD) of CREB-binding protein confirmed native-like induced folding upon binding to NCBD. Thus, the synthetic approach described herein can be useful for the preparation of various post-translationally modified analogues of TAD-p53 for further functional biochemical and biophysical studies.

1. Introduction

Tumor protein 53 (Tp53 or p53) continued to be a subject of active research since its first identification in the late 1970s.[1-10] The protein p53 first thought to be an oncoprotein has eventually emerged as a tumor suppressor playing central role in the maintenance of genome stability.[1,11] In unstressed cells, the negative regulator human double minute 2 (HDM2) protein polyubiquitinates p53 leading to its continuous proteosomal degradation, therefore, keeping p53 concentration at a low level.[12-14] Whereas, upon cell stress or DNA damage, p53 gets activated via disruption of the p53-HDM2 interaction, which results in accumulation of p53 leading to inhibition of cell growth and apoptosis.[12-14] In many human cancer cells p53 behaves incorrectly with up to 50% of tumors containing cells possessing p53 mutations.[11] Furthermore, cancer-inducing viruses can inactivate p53, for example, E6 protein of human papilloma virus (HPV) induces p53 degradation.[15]

The protein p53 consists of five major domains, such as transactivation domain (TAD), proline-rich domain, DNA binding core domain, tetramerization domain and regulatory domain.[13] The N-terminal TAD is acidic, intrinsically disordered and responsible for binding to various transcription factors.[13] The activity of p53 is controlled by its interaction with HDM2 and transcriptional co-activators CREB-binding protein (CBP) and p300.[14] The TAD of p53 has binding sites to interact with transcription adaptor putative zinc finger 1 (TAZ1), TAZ2, kinase-inducible domain (KID) interacting domain (KIX), and nuclear receptor co-activator binding domain (NCBD) of CBP or p300 along with the p53-binding domain of HDM2.[14] TAD-p53 interacts via its two active regions AD1 (1-42) and AD2 (43-63).[2] In the unstressed cells, AD2 binds to CBP domains while AD1 binds to HDM2.[14] Thus, through AD1 and AD2, TAD forms a ternary complex that leads to the degradation of p53 by ubiquitination.[14] On the other hand, genotoxic stress results in the phosphorylation at Thr and Ser residues of AD1 which severely weakens its interaction with HDM2,[14] which leads to preferential binding of TAD to the CBP domains and activation of p53.[13,14]

There are not many proteins in molecular oncology that have so many sites for post-translational modifications (PTMs) as p53.[16] Importantly, the tumor suppressor p53 was shown to be a phosphoprotein just after few years after its discovery.[17,18] Thus, phosphorylation turned into the most recognized PTM and was extensively studied over the years.[3] The TAD alone contains seven Ser and two Thr residues available for phophorylation.[14] A single phosphorylation at Thr18 results in two-times increase of p53 affinity towards TAZ1 of CBP.[14] A more significant 10-fold enhancement occurs when Ser15 and Ser20 are also phosphorylated along with Thr18.[14] Studies by several authors have shown that individual phosphorylations of Ser15, Ser20,

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Ser33, Ser37, Ser46, Thr18 and Thr55 modulate interactions of p53 to each of the four binding domains of CBP/p300 but to different extents.[16,19,20] However, Thr18 has been found having the most potent influence.[3,13,16] Moreover, it has been observed that depending on the type and intensity of the stress and cell type, differential phosphorylation and thereby fine modulation of p53-CBP/p300 interaction and transcriptional outcome occurs.[3,16] Thus, these events collectively provide a regulatory switch that facilitates TAD-p53 binding to CBP/p300 over its negative regulator HDM2.[16]

Apart from phosphorylation, there are several acetylation sites in the C-terminal domains of p53 which are crucial for p53 to maintain its function. The lysine residues, Lys305, Lys370, Lys372, Lys373, Lys381, Lys382, and Lys386 are acetylated during different forms of genotoxic and non-genotoxic stress.[16,21,22] Significance of acetylation lies in the fact that it is mutually exclusive to ubiquitination for p53 regulation.[16] In unstressed cells ubiquitination leads to the degradation of p53 while acetylation ensures non-ubiquitination of the p53 residues in stressed cells which leads to p53 stabilization. Thus, both phosphorylation and acetylation are critical factors for p53 stabilization.[16]

Although there are multiple studies that provided valuable insights into the role of phosphorylation of individual residues and other PTMs, there are still many questions that need to be addressed to fully understand the biological significance of p53. The TAD-p53 (wild-type or phosphorylated) used so far in most of the studies were either prepared biosynthetically or biologically expressed[14] or in some cases a small part of it has been synthesized chemically.[13] The efficient total chemical synthesis or semi-synthesis of full-length p53 would thus be particularly attractive to address the questions of the role of the combinations of PTMs in p53 function because such methods can allow for preparation of modified analogues with precise patterns of the desired modifications.[23] Towards this goal, in this manuscript a detailed synthetic procedure for preparation of TAD-p53 is described based on the combination of Fmoc/tBu-solid phase peptide synthesis (SPPS) and native chemical ligation (NCL), which complements previously published synthetic protocol.[24]

2. Results and Discussion

In this work the 61-residue transactivation domain of p53 (TAD-p53) has been synthesized using a three peptide segment ligation strategy (Fig. 1). The peptides were joined by native chemical ligation (NCL) using unprotected peptide-$^{\alpha}$thioesters and Cys-containing peptide segments.[25-29] The TAD-p53 has no native Cys residues in its sequence, therefore, Gln16 and Gln38 were replaced by Cys residues in which the thiol side chains were alkylated using bromoacetamide to achieve a side chain chemically similar to glutamine. Another modification was made to prevent the problem of oxidation of the methionines, which was shown to complicate the synthesis and isolation.[24] Thus, Met1, Met40 and Met44 were replaced with nearly isosteric norleucine (Nle) residues. As a result, the three segments in the synthesis were Nle1-Ser15 (segment-1), Cys16-Ser37 (segment-2) and Cys38-Asp61 (segment-3) (Fig. 1).

The ligation of three segments can be advantageous compared to previously reported synthesis based on a two-segment approach[24] for the following reasons: (i) peptide segments needed for ligation are shorter and easier to synthesize; (ii) elucidation of the role of PTMs requires synthesis of protein libraries where the three-segment strategy is more modular and flexible. PTMs can occur along the entire sequence of TAD-p53.[3] For this reason larger combinatorial diversity of the library will be easier to achieve with three-segment ligation approach, which will require lesser number of peptide building blocks. For example, to prepare all 512 possible phospho-variants of TAD-p53 (seven Ser and two Thr phosphorylation sites lead to 2^9 = 512 combinations), it will require synthesis of 132 peptide segments (2^7+2^2) via a two-segment ligation strategy (approach in ref. 24) and only 28 peptide building blocks ($2^3+2^4+2^2$) using a three-segment ligation method (Fig. 1).

The ligation junctions (residues (15-16) and (37-38), respectively) were chosen in the regions of the sequence that remain unstructured in the known complex with nuclear co-activator binding domain (NCBD) of CBP,[2] therefore modification of Gln16 and Gln38 into pseudo-glutamines is unlikely to affect the properties of the synthetic TAD-p53.

Both the C-to-N and N-to-C one-pot ligation strategies were tested in our study with different thioesters of the middle segment used in the N-to-C native chemical ligation and their comparative yields are presented in this article.

2.1. Synthesis of the peptide segments

For the synthesis of the segment-1 (Nle1-Ser15), manual conventional Fmoc/tBu-SPPS was applied using hydrazide resin and HBTU (hexafluorophosphate benzotriazole tetramethyl uronium) as coupling agent and *N,N*-diisopropylethylamine (DIEA) as activator base (details are in the *Appendix A*). Subsequent cleavage from the resin and HPLC purification resulted in a yield of 56%. Segment-1 (Nle1-Ser15)-$^{\alpha}$NHNH$_2$ was then transformed into the corresponding $^{\alpha}$thioester (Fig. S1). To achieve such conversion, the C-terminal $^{\alpha}$hydrazide functional group was first oxidized to $^{\alpha}$azide by sodium nitrite and *in situ* thioesterified with MeSNa (sodium 2-mercaptoethanesulphonate) making it suitable for native chemical ligation.[30]

Meanwhile, the middle segment-2 (Cys16-Ser37) was synthesized in two forms (see Table S1 and Fig. S2). In the first approach, using the hydrazide resin, (Cys16-Ser37)-$^{\alpha}$NHNH$_2$ (segment-2A) was synthesized using microwave-assisted SPPS using *N,N′*-diisopropylcarbodiimide (DIC) and Oxyma (2-cyano-2-(hydroxyimino)acetic acid ethyl ester) as coupling reagents. After cleavage and HPLC purification the yield was found to be 32%. Such (Cys16-Ser37)-$^{\alpha}$NHNH$_2$ containing *N*-terminal Cys residue was used in N-to-C ligations.

In another method, employed for C-to-N ligations, *N*-acylurea linker was used for the middle segment synthesis.[31] In this process, a linker 3-amino-4-(methylamino)benzoic acid was prepared with the primary amino group Fmoc-protected.[31] The Fmoc-Rink amide resin was used in this case and amino acid glycine was coupled to it followed by the linker and then the *C*-terminal amino acids starting from Ser37 were coupled using Fmoc-SPPS. As required in this method the last coupled *N*-terminal amino acid was Boc-Thz (Boc-thiazolidine).[31] It has to be noted that in the synthesis of (Thz16-Ser37)-$^{\alpha}$MeNbz (segment-2B) a more efficient coupling reagent HATU (hexafluorophosphate azabenzotriazole tetramethyl uronium) needs to be used instead of HBTU in conjunction with double couplings of the five C-terminal residues to avoid deletions. The peptide was prepared at room temperature using Syro I automatic peptide synthesizer. After the last coupling, the *N*-methylaniline

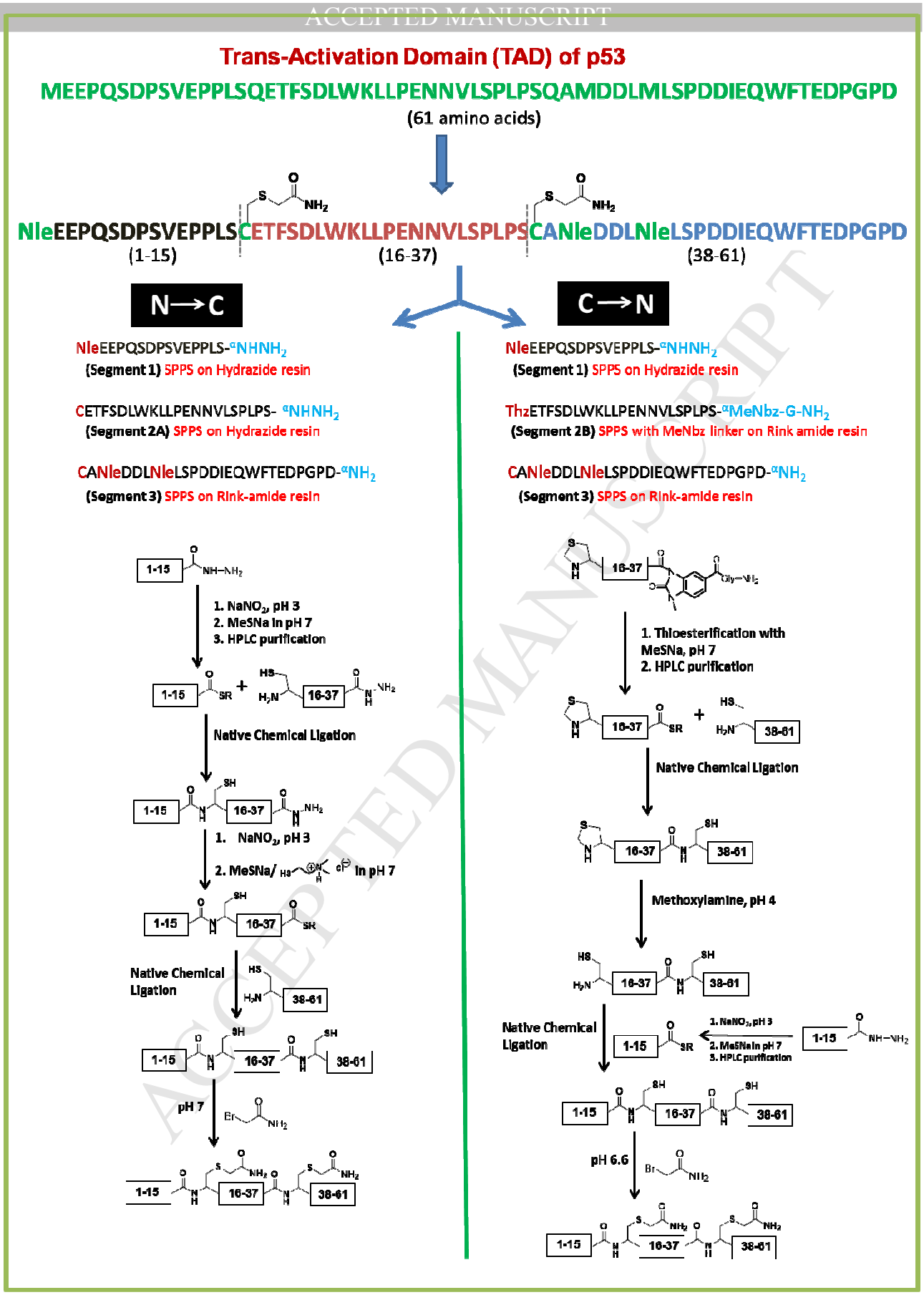

Fig. 1. Schematic representation of the N-to-C and C-to-N peptide ligation strategies for the total chemical synthesis of transactivation domain (TAD) of p53.

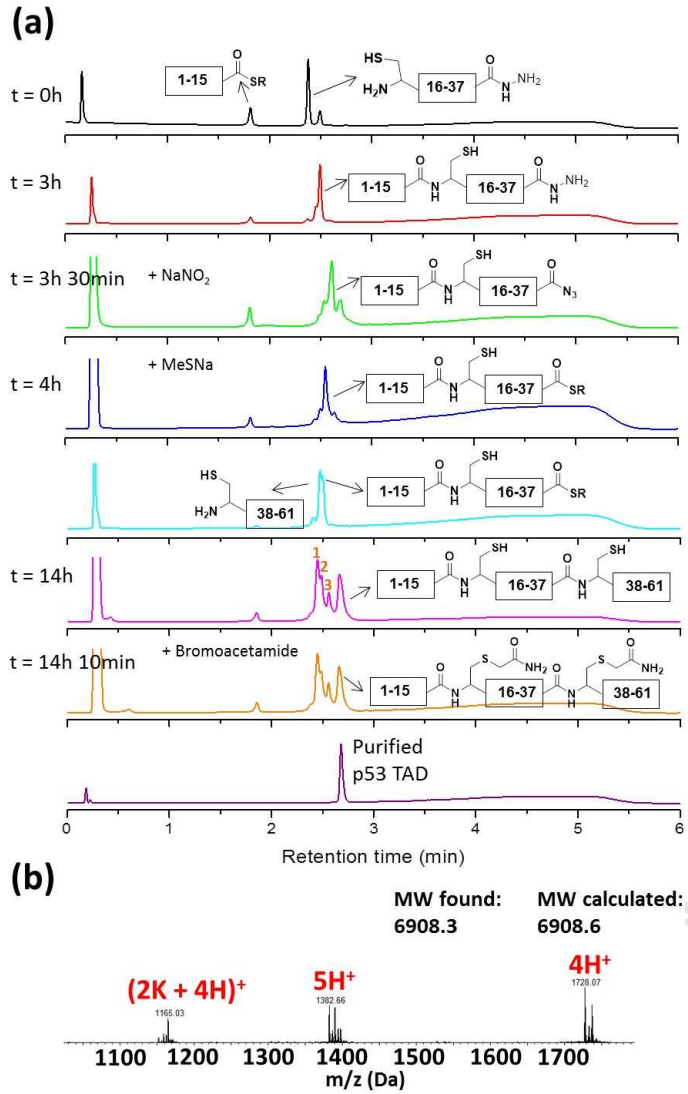

Fig. 2. (a) One-pot native chemical ligation towards TAD-p53 in N-to-C direction (using MeSNa for preparing active thioester after first NCL) monitored by analytical HPLC. By-products in panel 6 (from the top) are (1) (Cys38-Asp61)-$^{\alpha}$amide, (2) (Nle1-Ser15)-(Cys16-Ser37)-$^{\alpha}$OH, (3) (Nle1-Ser15)-(Cys38-Asp61)-$^{\alpha}$amide. (b) ESI-MS-Orbitrap analysis of the pure TAD p53. Multiple peaks for each charge state are due to Na^+ and K^+ ion adducts. A repeating of the synthesis (summarized in Fig. S4) using this procedure with several modifications (see *Appendix A*) confirms complications in N-to-C strategy.

moiety (linker) was subjected to cyclization on resin using chloroformate derivative and DIEA base in successive steps to convert it into an *N*-acylurea (MeNbz) moiety.[31] The peptide was cleaved and purified with a yield of 25% before MeSNa was used for thioesterification of (Thz16-Ser37)-$^{\alpha}$MeNbz by substituting the MeNbz moiety (Fig. S2). The resultant (Thz16-Ser37)-$^{\alpha}$thioester building block was used for C-to-N ligations.

The C-terminal (Cys38-Asp61)-$^{\alpha}$amide segment was synthesized on a Rink-amide resin using microwave-assisted SPPS using DIC and Oxyma with an isolated yield of 16% (Fig. S3) and was used in both N-to-C and C-to-N ligations.

2.2. Native chemical ligation of peptide segments

2.2.1. N-to-C one-pot and sequential ligations

The two segments, (Nle1-Ser15)-$^{\alpha}$SR (MeSNa thioester) and (Cys16-Ser37)-$^{\alpha}$NHNH$_2$ were ligated in a denaturing buffer (6 M guanidine-HCl, 200 mM phosphate, pH 7) within 3 h with a good conversion as evident from analytical HPLC (Fig. 2). Subsequently, (Nle1-Ser15)-(Cys16-Ser37)-$^{\alpha}$NHNH$_2$ was converted to $^{\alpha}$thioester upon treatment with $NaNO_2$ and *in situ* reaction with MeSNa furnishing the corresponding (Nle1-Ser15)-(Cys16-Ser37)-$^{\alpha}$thioester ready for the second NCL with (Cys38-Asp61)-$^{\alpha}$amide segment. However, we found that the second NCL was complicated by hydrolysis of (Nle1-Ser15)-(Cys16-Ser37)-$^{\alpha}$thioester. The reaction was continued for extended period of time (10 h) to reach maximum possible conversion. In Fig. 2 (6th panel from the top) the peaks **1**, **2**, **3** correspond to unreacted (Cys38-Asp61)-$^{\alpha}$amide, (Nle1-Ser15)-(Cys16-Ser37)-$^{\alpha}$OH and (Nle1-Ser15)-(Cys38-Asp61)-$^{\alpha}$amide, respectively, along with the desired (Nle1-Ser15)-(Cys16-Ser37)-(Cys38-Asp61)-$^{\alpha}$amide product. High quantities of unreacted (Cys38-Asp61)-$^{\alpha}$amide are due to undesired depletion of (Nle1-Ser15)-(Cys16-Ser37)-$^{\alpha}$thioester. The by-product **3** arises from the undesired ligation between (Nle1-Ser15)-$^{\alpha}$SR (remaining from the first ligation) and (Cys38-Asp61)-$^{\alpha}$amide, which is present in excess.

The next step was the alkylation of the two Cys residues to convert them into pseudo-glutamines (ψ-Gln16 and ψ-Gln38). To achieve this, 1 M bromoacetamide was prepared in 6 M guanidine-HCl, 200 mM phosphate, pH 7 buffer and added to the reaction mixture in three-fold excess over total thiol amount in the solution and kept at 40 °C after adjusting the pH to 6.6. This reaction was fast and was stopped after 10 min since longer reaction times can result in undesirable alkylation of residues other than Cys. Reduction of pH to around 4 after the reaction prevents any undesired alkylation. Purification of the reaction mixture resulted in the final 61-residue TAD-p53 analogue in 13% overall yield (based on the (Nle1-Ser16)-$^{\alpha}$SR).

Instead of adding MeSNa to convert (Nle1-Ser15)-(Cys16-Ser37)-$^{\alpha}$azide into the corresponding $^{\alpha}$thioester, ligation with (Cys38-Asp61)-$^{\alpha}$amide was also tried with 50 mM or 100 mM MPAA (4-mercaptophenylacetic acid) through *in situ* thioester generation and subsequent ligation but in each case large amounts of hydrolyzed (Nle1-Ser15)-(Cys16-Ser37)-$^{\alpha}$OH were observed concomitant with minute amounts of the ligation product with (Cys38-Asp61)-$^{\alpha}$amide segment (data not shown). It appears that activation in the form of aryl-thioester produces highly reactive peptide-$^{\alpha}$thioester particularly labile to hydrolysis. In the presence of a different alkyl-thiol such as 2-(dimethylamino)ethanethiol hydrochloride used previously in NCL[32] introduced for transforming (Nle1-Ser15)-(Cys16-Ser37)-$^{\alpha}$azide into a thioester (while maintaining the same concentrations and conditions as of MeSNa) the desired full length TAD-p53 could be obtained in a 10% yield (based on (Nle1-Ser15)-$^{\alpha}$SR) which is similar to the results with MeSNa thiol (Fig. S5).

The sequential three segment ligation in N-to-C direction rather than one-pot procedure was also attempted. In this case the product of the first ligation (Nle1-Ser15)-(Cys16-Ser37)-$^{\alpha}$NHNH$_2$ was alkylated using 2-bromoacetamide and converted into the corresponding $^{\alpha}$thioester followed by HPLC purification. Subsequently, second ligation was performed (see Fig. S6). After Cys alkylation and second HPLC purification, the desired product was obtained, however, with the overall yield (6%) less

than half of what was obtained from one-pot procedure because of losses in the additional HPLC purification.

2.2.2. C-to-N one-pot ligation approach

To improve the efficiency of the sequential native chemical ligations and reduce the amount of by-products arising from the hydrolysis observed in the N-to-C ligation approach, a reverse C-to-N ligation route was adopted (Fig. 3). In this case the thioesterification of the middle segment was performed separately and unlike in N-to-C ligation the (Thz16-Ser37)-$^{\alpha}$SR (MeSNa thioester) segment was used as the starting peptide containing Thz (thiazolidine) moiety at the N-terminus instead of Cys, which is necessary to avoid undesired head-to-tail cyclization. Thus, (Thz16-Ser37)-$^{\alpha}$SR (MeSNa thioester) and (Cys38-Asp61)-$^{\alpha}$amide were ligated in 6 M guanidine-HCl, 200 mM phosphate buffer at pH 6.8 (Fig. 3). Subsequently, the thiazolidine ring was cleaved by treatment with 0.2 M methoxyamine-HCl at pH 4. After thiazolidine deprotection, second ligation with (Nle1-Ser15)-$^{\alpha}$thioester segment was performed in the presence of 50 mM MPAA. The MPAA aryl-thiol catalyst was used to increase the efficiency of the second ligation in the presence of an internal Cys residue.[28] It took 4 h at 40 °C for the reaction to reach near completion indicated by unchanged relative intensity of the analytical HPLC peaks (Fig. 3). Then, 2-bromoacetamide was used for alkylation similar to the procedure described earlier for N-to-C ligations. An overall yield of 26% was obtained after HPLC purification of the final peptide (based on (Cys38-Asp61)-$^{\alpha}$amide segment).

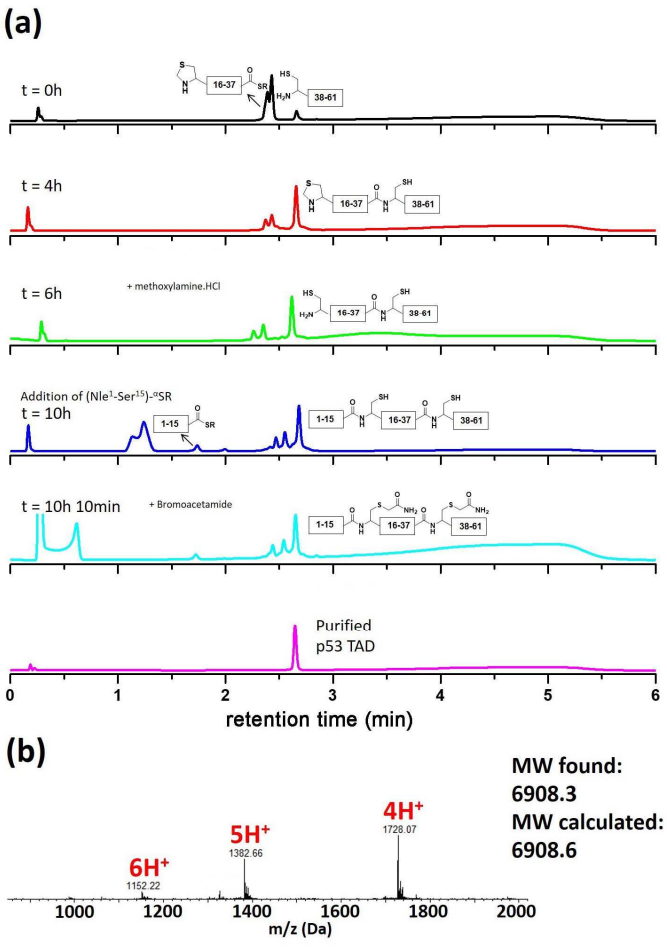

Fig. 3. (a) One-pot native chemical ligation of three peptide segments in C-to-N direction monitored by analytical HPLC. (b) ESI-MS-Orbitrap analysis of the pure TAD-p53.

2.3. Characterization of binding of chemically synthesized TAD-p53 to nuclear co-activator binding domain (NCBD)

2.3.1. Circular dichroism study

TAD-p53 is an intrinsically disorder protein domain, *i.e.* in isolated state it does not have well-defined secondary or tertiary structure.[13] We confirmed that synthetic TAD-p53 is unstructured, which can be seen in its CD spectrum corresponding to a random coil (Fig. 4). Intrinsic disorder plays a key role in the biological function of p53 by providing structural plasticity and enabling this protein to bind to a variety of other proteins.[33] In this way, p53 can serve as a hub in protein-protein interaction networks regulating gene expression.[33] One of such interacting partners is nuclear co-activator binding domain (NCBD) of CREB-binding protein (CBP). We therefore inspected structural changes in synthetic TAD-p53 which occur upon interaction with NCBD by recording the CD spectra of TAD-p53/NCBD complex. We found that the helicity in the complex increases significantly (Fig. 4) compared to free TAD-p53 or NCBD alone judged by the enhancement of ellipticity at 222 nm. The presence of α-helices in the TAD-p53/NCBD complex is in accordance with previously reported NMR structure of the complex.[2] Thus, the CD experiments confirm that the chemically synthesized TAD-p53 retains the characteristics of biologically expressed protein domain by forming a complex with NCBD.

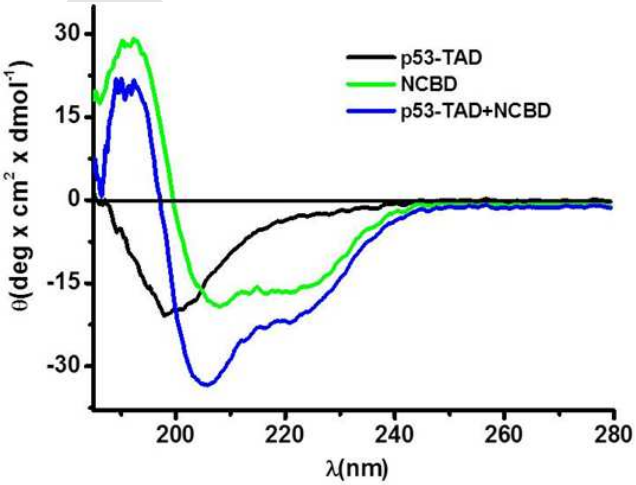

Fig. 4. Circular dichroism (CD) spectra of free TAD-p53, NCBD and their 1:1 complex in 20 mM phosphate buffer at pH 7.4 at 25 °C (concentration of protein = 25 µM).

2.3.2. NMR study

In addition, 1D ^{1}H NMR and 2D ^{1}H-^{13}C heteronuclear single quantum coherence (HSQC) spectroscopy were also used to monitor structural changes occurring upon binding of TAD-p53 to NCBD (Fig. 5). Changes upon binding are clearly visible in ^{1}H NMR corresponding to aromatic protons (spectra were recorded in D_2O), where sharp resonances are present for free TAD-p53, which is in agreement with intrinsically disordered properties of this protein domain, whereas signals become broader upon addition of NCBD corresponding to a larger size of the complex and different environment of aromatic side-chains (Fig. 5a). The ^{1}H-^{13}C HSQC is also supportive of complex formation: overlay of the methyl regions of the spectra for free TAD-p53 and its complex with NCBD show distinct resonances indicative of different chemical surroundings of the corresponding methyl groups (Fig. 5b).

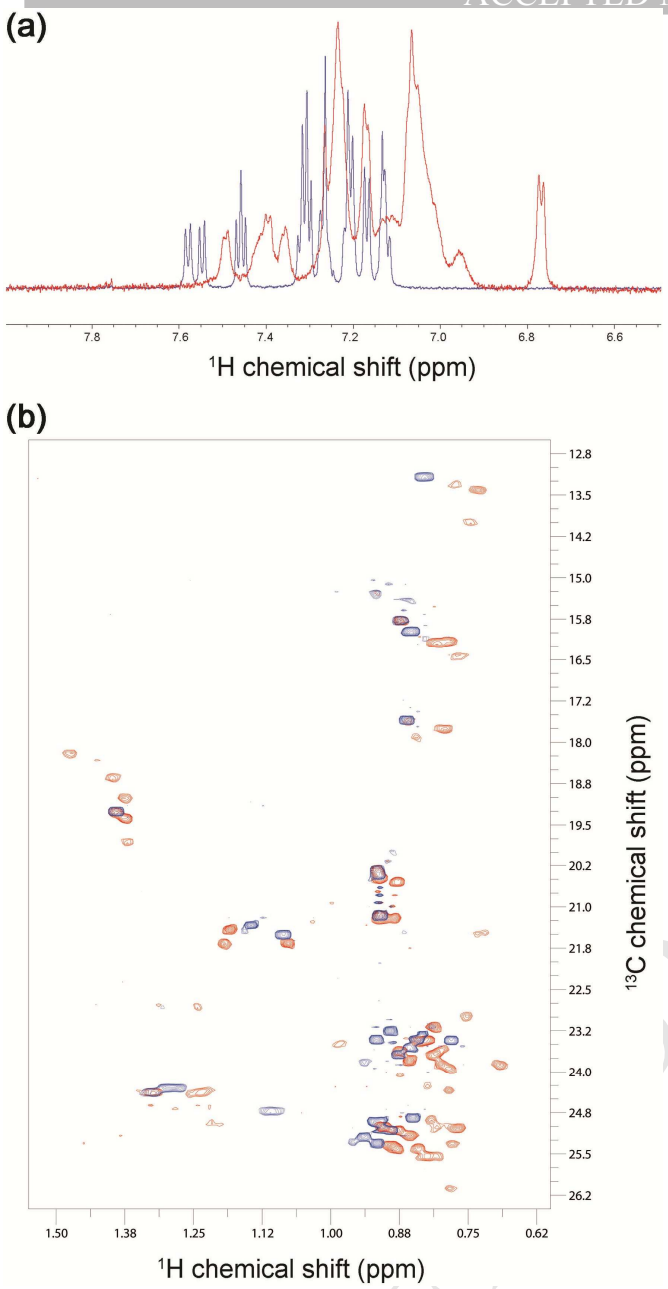

Fig. 5. (a) Overlay of ^{1}H NMR (aromatic region) and (b) ^{1}H-^{13}C HSQC (methyl region) spectra of free TAD-p53 (in blue) and 1:1 complex with NCBD (in red) recorded in D_2O.

3. Conclusions

In this article we reported total chemical synthesis of the (1-61)-form of transactivation domain (TAD) of p53 protein based on native chemical ligation of three peptide segments. The peptide building blocks are relatively short (less than 24 residues) and can be efficiently synthesized using Fmoc/tBu-SPPS approach accessible in many academic laboratories using commercially available building blocks. Two approaches, N-to-C and C-to-N have been used to ligate the peptide segments to achieve the full-length TAD-p53 and it has been found that C-to-N approach results in 2- to 2.5-fold higher yield than N-to-C method. The non-canonical residues introduced in the sequence to facilitate the synthetic protocols such as three norleucines (at sites 1, 40 and 44) and two pseudo-glutamines (at sites 16 and 38) do not affect coupled folding and binding to NCBD which is a known binding partner of p53. The reported synthetic approach based on the ligation of three segments will be particularly advantageous for the combinatorial synthesis of the post-translationally modified variants of TAD-p53 (*e.g.* phosphorylated) in order to perform proteome-wide interaction profiling as a function of phosphorylation pattern using protein arrays and other approaches to study protein binding interactions.

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Appendix A. Supplementary data

Detailed experimental procedures and characterization of synthesized compounds can be found as the supplementary data related to this article at https://

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