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Convergent solid-phase and solution approaches in the synthesis of the cysteine-rich Mdm2 RING finger domain

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The RING finger domain of the Mdm2, located at the *C*-terminus of the protein, is necessary for regulation of p53, a tumor suppressor protein. The 48-residues long Mdm2 peptide is an important target for studying its interaction with small anticancer drug candidates. For the chemical synthesis of the Mdm2 RING finger domain, the fragment condensation on solid-phase and the fragment condensation in solution were studied. The latter method was performed using either protected or free peptides at the *C*-terminus as the amino component. Best results were achieved using solution condensation where the *N*-component was applied with the *C*-terminal carboxyl group left unprotected. The developed method is well suited for large-scale synthesis of Mdm2 RING finger domain, combining the advantages of both solid-phase and solution synthesis. Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.

Supporting information may be found in the online version of this article

Keywords: Mdm2; RING finger domain; convergent synthesis; solution synthesis; solid-phase peptide synthesis; Clt resin

Introduction

The tumor suppressor protein p53 plays a pivotal role in DNA damage recognition [1], signal transduction, and initiation of mechanisms of apoptosis and repair [2]. Mdm2 protein interacts with p53, inhibits its function and promotes its degradation through ubiquitination (programmed protein degradation) [3]. In the ubiquitin pathway, Mdm2 plays the role of an E3 ubiquitin ligase [4]. High levels of Mdm2 are associated with cancer initiation and, therefore, it is characterized as an oncogenic protein. It has been found that the RING domain of Mdm2 (Figure 1) located at the *C*-terminus of the protein, is necessary for E3 activity and, therefore, for the regulation of p53 [5]. Thus, there is a great interest in understanding the interaction the RING domain of Mdm2 with anticancer drug candidates.

The synthesis of such multiple cysteine residues-containing small proteins is difficult to achieve using recombinant techniques, due to their high tendency to form aggregates [6,7]. Therefore, chemical syntheses are attractive and in two examples the ligation method was used [8,9]. Ligation, as it is currently carried out, is very efficient for the preparation of peptides in small quantities (<100 g). However, the ligation method at larger scale is challenging since it requires that all peptide intermediates be cleaved from the resin, deprotected using HF and purified by HPLC. For example, the preparation of one peptide by chemical ligation requires at least two unprotected fragments and three HPLC-purifications. As a result, the production cost increases considerably and is in many cases economically unacceptable for the development of a pharmaceutical product. As an alternative approach, we studied the convergent synthesis of the 48mer RING finger domain of Mdm2 (sequence 435–482), by sequential condensation of protected fragments either on Clt resin or in solution.

The main advantage of the fragment condensation strategy [10–18] is that a greater control of the synthesis is achievable. Fragments are synthesized in a stepwise manner using Clt resin and Fmoc-amino acids. During the synthesis, all intermediates can be analyzed with TLC, HPLC, and mass-spectrometry. The peptides can be cleaved from the resin under very mild conditions without affecting side-chain protection. Therefore, side-products of coupling and Fmoc-removal can be identified without influencing the side-chain protecting groups. Required double couplings and truncated sequences can be easily detected and measures for the elimination of difficult couplings can be taken.

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Abbreviations used: *AcN, acetonitrile; AcOH, acetic acid; Boc, tert-butyloxycarbonyl; Clt, 2-chlorotrityl; CLTR, 2-chlorotrityl chloride resin; DBU, 1,8-diazabicyclo[5.4.0]-undec-7-ene; DCM, dichloromethane; DEE, diethylether; DIC, N,N′-diisopropylcarbodiimide; DIEA, N,N-diisopropylethylamine; DMSO, dimethyl sulfoxide; DTT, 1,4-dithio-DL-threitol; EDC, 1-ethyl-3-[3-dimethyl-aminopropyl]carbodiimide hydrochloride; ESI-MS, electrospray ionization mass-spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; HBTU, O-benzotriazolyl-N,N,N′,N′-tetramethyluronium hexafluorophosphate; HF, hydrogen fluoride; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; i-PrOH, isopropanol; Mdm2, murine double minute chromosome clone number 2; MeOH, methanol; Mmt, 4-methoxytrityl; NMP, N-methyl-2-pyrrolidinone; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; RING, really interesting new gene; RP, reverse phase; RT, room temperature; tBu, tert-butyl; TES, triethylsilane; TFA, trifluoroacetic acid; TFE, trifluoroethanol; THF, tetrahydrofurane; TLC, thin layer chromatography; Trt, triphenylmethyl trityl.*

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H-I[1]-E-P-C-V-I-C-Q-G-R-P-K-N-G-C-I-V-H-G-K-T-G-H-L-M-A-C-F-T-C-A-K-K-L-K-K-R-N-K-P-C-P-V-C-R-Q-P-I[48]-OH **1**

Figure 1. Amino acid sequence of RING finger domain of Mdm2.

Corresponding optimizations allow a simple standardization of synthetic procedures. As a result, the scale up chemistry differs little from the small scale experiments.

Table 1. Experimental data of the resin-bound fragments **2**–**5**

Resin-bound fragment	Substitution (mmol/g)	HPLC[a] purity (%)	Main impurities[b] (%)	Mol. weight (Da)	
				Calcd.	Found
2	0.165	91	A (6), B -	2130.65	2130.64
3	0.139	82	A (13), B -	2784.48	2784.44
4	0.123	71	A (12), B (7)	3237.06	3236.57
5	0.117	74	A (10), B (6)	3523.39	3524.08

[a] HPLC analysis was performed after cleavage from the resin and side-chain deprotection; conditions E.
[b] A = *t*-butylation of Cys residues, B = Met^{25} sulfoxide.

Materials and Methods

Clt resin, Fmoc-amino acids, DIC, HOBt, and TFA were obtained from CBL-Patras (Patras, Greece). Solvents and other reagents used were purchased from Aldrich (Steinheim, Germany) and Merck (Darmstadt, Germany) and were of analytical grade. Acetonitril used for HPLC was of gradient grade (Merck). TLC-analyses: TLC-aluminium sheets, silica gel 60, F_{254} (Merck). RP-HPLC: Waters 600E multisolvent delivery system, combined with Waters 996 photodiode array detector and Alliance Waters 2695 Separations Module multisolvent delivery system combined with Waters 2996 photodiode array detector. Protected peptides **6**–**19** were analyzed on a C-8 Purospher column (particle size 5 µm, 125 × 4 mm) using linear gradients of 20–100% B over 30 min (conditions A), 40–100% B over 30 min (conditions B) or 70–100% B over 30 min (conditions C). Free peptides were analyzed on a C-18 Nucleosil-100 column (particle size 5 µm, 250 × 5 mm) using linear gradients of 20–70% B over 30 min (conditions D), 30–80% B over 30 min (conditions E) or 20–40% B over 30 min (conditions F). Eluent A was 0.08% TFA in water and eluent B was 0.08% TFA in AcN. Flow rate, 1 ml/min and UV detection at 214 and 265 nm. Purifications were carried out on a semipreparative C-18 Nucleosil column (particle size 7 µm, 250 × 10 mm) at a flow rate of 5 ml/min and UV detection at 225 nm, using linear gradients of 25–35% B in 40 min. ESI-MS: Waters Micromass ZQ, controlled by the MassLynx 4.1 software, cone voltage 30V.

Table 2. Experimental data of the protected fragments **6**–**19**

Fragment	Cleavage yield (%)	HPLC purity (%)	Conditions[a]	Mol weight (Da)	
				Calcd.	Found
6	72	98	C	1594.54	1593.97
7	80	n.d.	C	2289.10	2289.09
8	64	96	A	934.80	934.58
9	60	94	A	950.80	950.53
10	75	96	B	1706.64	1706.75
12	70	95	B	1264.18	1263.70
14	78	95	B	2853.86	2853.98
16	83	95	B	2411.40	2411.38
18	83	99	B	1387.47	1386.76
19	88	99	C	1902.72	1902.84

n.d., not determined.
[a] Column: C-8 Purospher (125 × 4 mm), for conditions A, B, C see Materials and Methods.

Resin Loading

CLTR was swollen with DCM (10 ml/g resin), to which DIEA (4 mmol/g resin) and the Fmoc-amino acid were added. After stirring for 2 h, MeOH (1 ml/g resin) and DIEA (2 mmol/g) were added to the reaction mixture for another 30 min. The resin was filtrated under vacuum and washed sequentially with DCM (2 × 1 min), DCM/MeOH/DIEA (85 : 10 : 5) (4 × 10 min) and NMP (10 × 1 min). The Fmoc-group was subsequently removed by treating the resin with 25% piperidine in NMP (1 × 5, 1 × 10, and 1 × 15 min) and washing with NMP (10 × 1 min).

General Procedure for Solid-phase Assembly of Protected Fragments

All syntheses were performed manually using plastic syringes equipped with porous polypropylene frits. The Fmoc-amino acids used for the chain assembly were protected at their side-chains with tBu for Glu and Thr, Trt for His, Asn and Gln, Boc for Lys, Pbf for Arg and Mmt for Cys. Coupling reactions were performed in NMP, by *in situ* activation of a threefold molar excess of Fmoc-amino acid/DIC/HOBt (1 : 1.1 : 1.5) at a concentration of 0.6 M for 4 h at RT, with the exception of the first coupling where HBTU/HOBt/DIEA (3 : 3 : 6) in NMP was used. The completion of the couplings was verified with the Kaiser test. The Fmoc protective group was removed by sequential treatment of the resin-bound peptide with 25% piperidine in NMP (1 × 5, 1 × 10, and 1 × 15 min). The completion of Fmoc-cleavage was verified using TLC by releasing the peptide from an aliquot of resin with the cleavage mixture DCM/TFE/AcOH (7 : 2 : 1). After each coupling and Fmoc-deprotection step, the resin was washed with NMP (10 × 1 min). Final peptide-resins were washed sequentially with NMP (10 × 1 min), i-PrOH (4 × 2 min), *n*-hexane (3 × 2 min) and left to dry under vacuum. Peptide fragments **2**–**5** were prepared using a starting loading of 0.4 mmol/g resin. Fragments **6**–**19** were prepared using a starting loading of 0.6 mmol/g resin. Table 1, summarizes the analytical data of the resin-bound fragments **2**– **5**.

General Procedure for the Cleavage of Protected Fragments from Resin

Fully protected fragments were cleaved from the CLTR by treatment with DCM/TFE (70 : 30) (15 ml/g resin) for 2 h at RT. The resin was removed by filtration under vacuum and washed twice with DCM. The combined filtrates were concentrated on a rotary evaporator and the protected peptide was precipitated by the addition of prechilled DEE, collected by filtration and dried under vacuum. Table 2 summarizes the analytical data of the protected fragments **6**– **19**.

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Global Deprotection

Side-chain deprotection was performed by treatment of the protected peptide with the cleavage cocktail TFA/DCM/TES/DTT (85 : 5 : 5 : 5) (1 ml/10 mg) for 1 h at 0–4 °C and 3 h at RT. The mixture was concentrated on a rotary evaporator or under a stream of N_2(depending on the volumes) and the fully deprotected peptide was precipitated by the addition of prechilled DEE, collected by filtration or centrifugation and left to dry to a constant weight.

Solid-Phase Fragment Condensation

According to the final substitution of each resin-bound fragment, a twofold molar excess of Fmoc-protected fragment, preactivated with DIC/HOBt (2.2 : 3), was applied in DMSO at a concentration of 0.15 M to the previously swelled peptide-resin and incubated for 12 h at RT. Aliquots of the resin were removed from the reaction mixture to verify coupling completion by Kaiser test. If the test was positive (blue-purple color), the peptide-resin was washed with DMSO (10 × 2 min) and a second condensation was performed under the same conditions. Remaining unreacted amino functions were capped with Boc-dicarbonate (10 eq.) and DIEA (5 eq.) in NMP. Finally, the resin suspension was filtrated and washed with NMP (10 × 1 min). All condensation products were analyzed, after deprotection, by RP-HPLC and identified by ESI-MS.

Fragment Condensation in Solution

Protocol A. Application of N-component as Clt ester

Fmoc-protected *N*-component **28** (36.7 mg, 6 μmol) was converted to the corresponding Clt ester **29** by reaction with Clt-Cl (5.6 mg, 18 μmol) and DIEA (6 μl, 36 μmol,) in DCM (400 μl). The reaction mixture was stirred for 6 h at RT. The solution was concentrated under a stream of N_2 and the protected Clt-peptide ester was precipitated by the addition of prechilled DEE and collected by centrifugation. The Fmoc-protective group was removed by treatment of the solid material with 2% DBU in NMP (200 μl) for 40 min at RT. The product **30** was isolated by centrifugation after precipitation with cold DEE, washed with cold DEE and, dried under vacuum. A portion of this material (24.67 mg, 4 μmol), Boc-protected *C*-component **25** (19.9 mg, 4.4 μmol) and HOBt (0.77 mg, 5.72 μmol) were dissolved in NMP (200 μl) at RT. After 5 min, DIC (0.75 μl, 4.84 μmol) was added and the mixture was stirred for 6 h at RT. Addition of DEE in the reaction mixture afforded the crude peptide **31** as a white precipitate. The Clt-group was removed by dissolving **31** in TFE/DCM (30 : 70) (300 μl) and stirring for 1.5 h at RT. The obtained product was subsequently totally deprotected by TFA/DCM/TES/DTT (85 : 5 : 5 : 5), as described above, to provide crude **1**. After semipreparative RP-HPLC, 5 mg (24%) of purified **1** were obtained.

Protocol B. Application of N-component as free carboxylic acid

Boc-protected *C*-component **27** (17.9 mg, 4.4 μmol) and HOBt (0.59 mg, 4.4 μmol) were dissolved in DCM (200 μl) and the solution was cooled to 0–4 °C. EDC (0.84 mg, 4.4 μmol) was added and the mixture was stirred for 20 min at 4 °C and 20 min at RT. The solution of the preactivated fragment was then added drop-wise into a stirred solution of protected *N*-component **35** (25.3 mg, 4.0 μmol,) in DCM (200 μl). The reaction mixture was incubated for 2 h at RT. DCM was removed under a stream of N_2 and the product mixture was precipitated by addition of cold water, isolated by centrifugation and purified by semipreparative RP-HPLC. After total deprotection, 7.8 mg (37%) of **1** was obtained.

Results and Discussion

The selection of fragments plays a crucial role in effectively synthesizing a peptide by fragment condensation. The most important factors which must be taken into consideration include susceptibility of racemization, solubility, and reactivity. Fragments containing Gly, Pro [10,11], or pseudoprolines [19,20] as the *C*-terminus are preferred because they do not racemize. Amino acids prone to racemization, such as cysteine and histidine, must be avoided. In addition, the condensation efficiency of the fragments depends on the *N*-terminal amino acid of the amino component to which they are being coupled. Sterically hindered amino acids must be avoided if possible [10,11]. Alternatively the *C*-terminal fragment can be applied in solution. In this case, its synthesis is favorably performed on solid-phase and protected, if necessary, at the *C*-terminus with an appropriate group, such as Clt, trityl, or tBu.

To optimize peptide synthesis by the fragment condensation approach, a stepwise assembly is carried out as to establish difficult synthetic regions. Efforts can then be taken to overcome the problems associated with the fragment condensation on solid-phase. Typically, the *C*-component is applied in excess in order to complete condensation reactions. For larger scale syntheses, applying the *C*-component in excess is not economical because the fragments are nonrecoverable. Therefore, the solid-phase condensation synthesis is a guide for selecting the correct sequences for carrying out the condensation reactions in solution where equimolecular quantities are used.

Five-Fragment Approach Starting from the Resin-bound 33–48 Peptide

We started our studies by preparing the resin-bound 33–48 protected peptide **2** according to Figure 2. To this peptide, we planned to condense the solid-phase synthesized protected fragments 23–32 (**7**), 15–22 (**10**), 10–14 (**18**), and 1–9 (**19**) (Figure 3). In these syntheses, the cysteine residues were protected with the Mmt group [21], a very acid sensitive protecting group. Mmt, in contrast to Trt, is easily and irreversibly removed by mild acidic treatment. DIC/HOBt was used as the condensing agent and 25% piperidine in NMP for the Fmoc-removal. The protected fragments **7, 10,18**, and **19** were obtained after cleavage from the resin with a 1 h treatment at RT with a 30 : 70 mixture of TFE/DCM. Under these conditions other side-chain protecting groups were unaffected.

Peptide **2** was synthesized without difficulty in high purity (91%), as revealed by HPLC (Figure 4(a)). Despite the susceptibility of cyclization in the sequence Pro–Ile, the *C*-terminal dipeptide, no diketopiperazine was observed with the use of the hindered Clt resin-ester [12,22–24].

In contrast, synthesis of the fragment **7** proved to be difficult after the incorporation of Ala[26]. Due to its insolubility, fragment **7** was eluted as a very broad peak in RP-HPLC, as shown in Figure 5(a). By combining the ESI-mass spectrum from 12 to 18 min it was found that more than 50% unresolved des-Met[25] peptide was present (Figure 5(b)), indicating that the Fmoc-removal at this position was incomplete even after 2 h treatment with piperidine. This region represents a *difficult* sequence of the RING domain and the evaluation of fragment 23–32 was discontinued.

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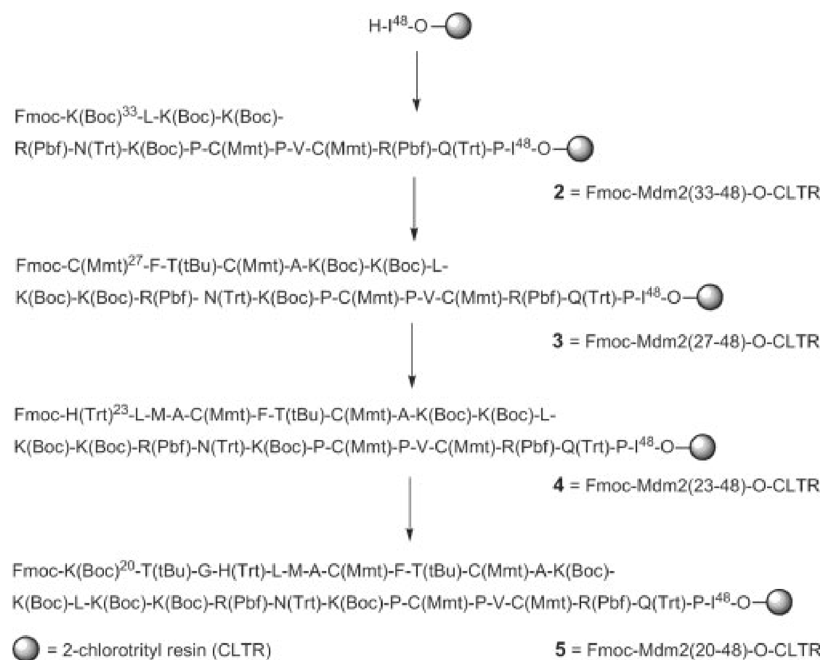

Figure 2. Solid-phase synthesis of the resin-bound amino components **2**–**5** of the RING finger domain of Mdm2.

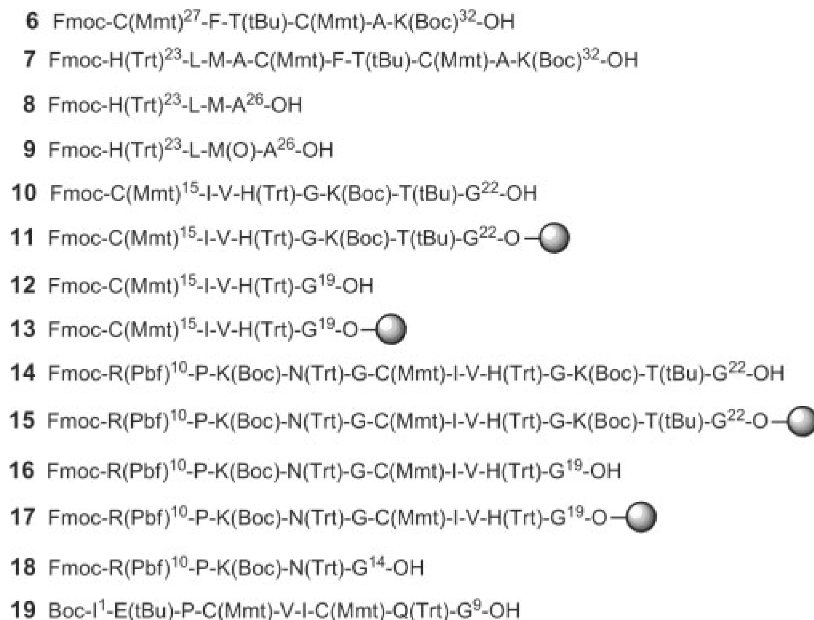

Figure 3. Protected fragments used in the convergent synthesis of the RING finger domain of Mdm2.

Six Fragments Approach Starting from Resin-bound 33–48 Peptide

To avoid the problematic synthesis of the 23–32 peptide, the 27–32 peptide **6** and the 23–26 fragment **8** were selected. Following standard synthetic protocols both peptides were obtained in high purity (Table 2). Fragment condensation was carried out in DMSO using DIC/HOBt as condensing agent. Unfortunately, peptide **6** racemized considerably at Lys^{32} (20%) during its condensation with the 33–48 resin-bound fragment **2**. As depicted in Figure 6(a), we were able to separate the D-diastereomer by analytical HPLC. Although the D-diastereomer was separated by HPLC, this synthetic approach was not further investigated.

Five-Fragment Approach Starting from the Resin-bound 27–48 Peptide

Instead of optimizing the condensation conditions in order to minimize racemization at Lys^{32}, the sequence of the 33–48 peptide **2** was lengthened by the stepwise methodology to the 27–48 sequence **3**. Peptide **3** was obtained in a purity of 82% after side-chain deprotection, while 13% of by-products were *t*-butylation of Cys residues (Figure 4(b)). The 23–26 fragment **8** was condensed with the resin-bound 27–48 peptide. Again, a

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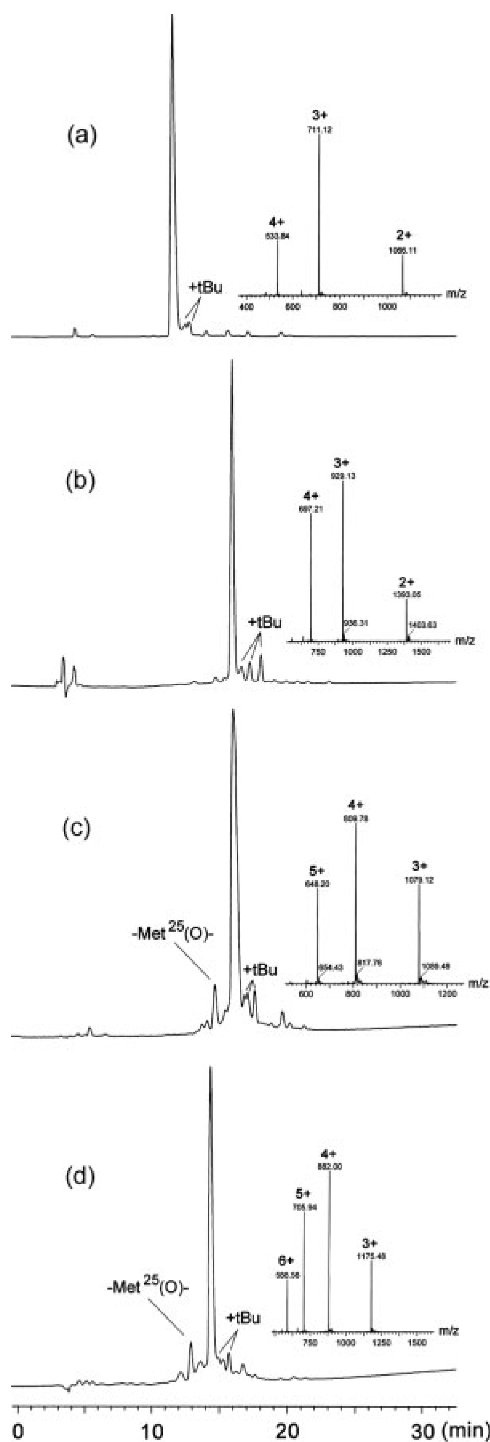

Figure 4. Analytical RP-HPLC of the crude products and ESI-MS of main components obtained after cleavage from the resin and side-chain deprotection of (a) **2**, (b) **3**, (c) **4**, and (d) **5** resin-bound fragments of Mdm2 RING domain; conditions E (Section Materials and Methods).

high degree of racemization was observed at Ala^{26} (22% D-isomer). For comparison, we also tested the corresponding Met-sulfoxide 23–26 peptide **9**, which was prepared via on-resin oxidation with 10% hydrogen peroxide in THF (Supporting Information). However, a similar degree of racemization was detected. Both D-isomers appeared as two distinct peaks in the HPLC-analysis of the crude deprotected peptides (Figure 6(b, c)).

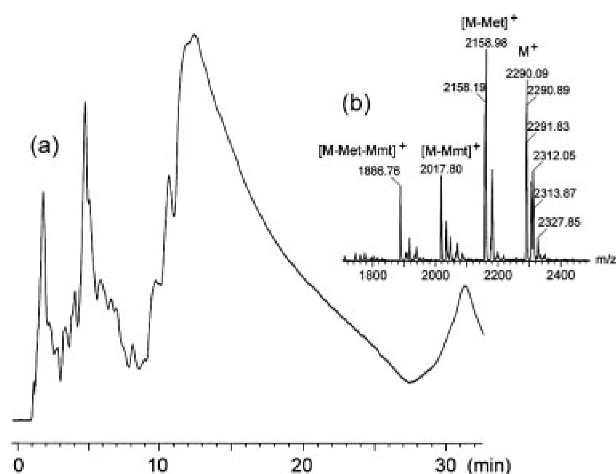

Figure 5. (a) RP-HPLC analysis of the protected 23–32 peptide **7**; conditions C and (b) combined ESI-MS from 12 to 18 min.

Four-Fragment Approach Starting from the Resin-bound 23–48 Peptide

A new synthetic approach starting from the stepwise elongated resin-bound 23–48 peptide **4** was attempted in order to avoid racemization at Ala^{26}. Interestingly, peptide **4** (Figure 4(c)) was obtained in a purity of 71% after side-chain deprotection, in contrast to peptide **7**. Therefore, resin-bound peptide **4** and the 15–22 fragment **10** were condensed, followed by the condensation of the fragments **18** and **19** (Figure 7). Although fragment **10** was applied in a 2.5 M excess and a recondensation was carried out after 24 h at RT, the reaction proceeded only to 55% completion, as shown in the analytical HPLC (Figure 8(a)), leading to a product with relatively low purity. Remaining unreacted amino functions were capped with Boc-dicarbonate. Boc-dicarbonate was preferred over acetic anhydride because the acetylated truncated sequence coeluted with the required product. Condensation of protected fragments **18** and **19** in 1.5 M excess proceeded without difficulties and went to completion within 12 h at RT. After the chain assembly, protected 1–48 peptide **22** was cleaved from the resin. The deprotection of this peptide was studied in the absence of the resin. This method avoids the contact of the free thiol groups of the Cys residues with resin-bound chlorotrityl cations formed during the treatment with concentrated TFA-solutions, lowering the yield. Several deprotection mixtures were tested. The best mixtures were found to be TFA/DCM/TES (85 : 10 : 5), TFA/DCM/DTT (85 : 10 : 5) and TFA/DCM/TES/DTT (85 : 5 : 5 : 5), with the latter being slightly more effective, in terms of purity. Therefore, the obtained 1–48 peptide **23** was totally deprotected with TFA/DCM/TES/DTT (85 : 5 : 5 : 5) for 1 h at 0 °C and 3 h at RT to provide crude Mdm2 RING finger in 21% purity. Purification by semipreparative HPLC yielded 8% of **1**.

Four-Fragment Approach Starting from the Resin-bound 20–48 Peptide

Due to the low yield obtained starting from the 23–48 protected sequence, the resin-bound peptide was elongated to 20–48 peptide **5** (Figure 4(d)). To this resin-bound peptide, the 15–19 fragment **12** was condensed, followed by the condensation of the fragments **18** and **19**. A single coupling of fragment **12** in twofold molar excess in respect to the resin-bound 20–48 peptide proceeded within 24 h at RT to 77% (Figure 8(b)). The

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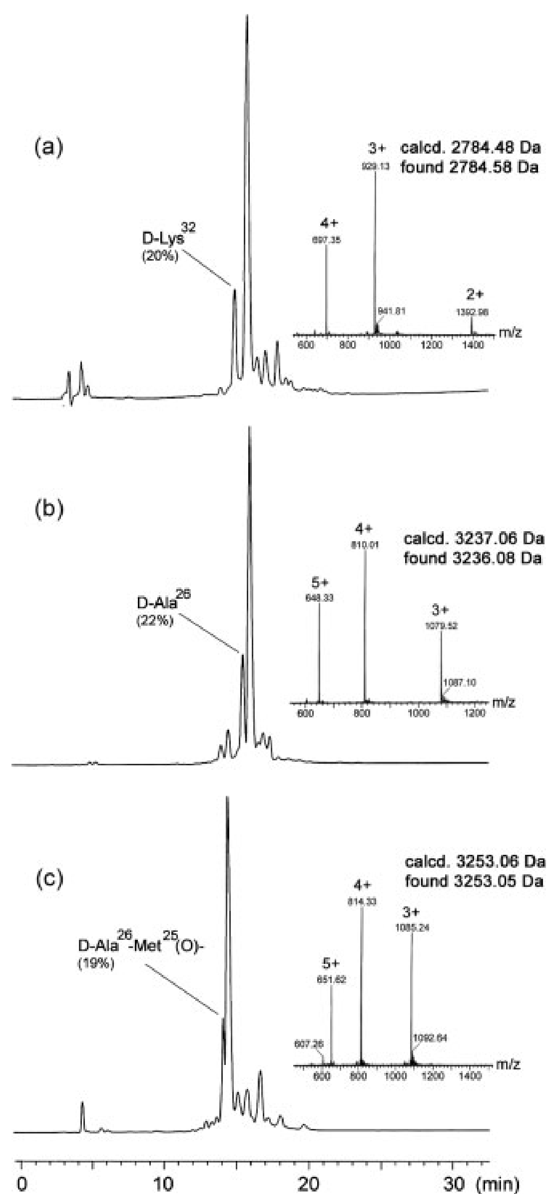

Figure 6. RP-HPLC analysis of the product mixtures obtained by solid-phase condensation of (a) Fmoc-(27–32)-OH with H-(33–48)-O-CLTR, (b) Fmoc-(23–26)-OH with H-(27–48)-O-CLTR, and (c) Fmoc-Met[25](O)(23–26)-OH with H-(27–48)-O-CLTR, after splitting from resin and side-chain deprotection; conditions E. The embedded ESI-MS shown in each case corresponds to the main component and to the preceding D-diastereomer.

remaining unreacted amino functions were capped with Boc-dicarbonate. Condensations with **18** and **19** proceeded similarly without difficulties. Deprotection and purification, as described above, gave Mdm2 RING finger in 15% yield.

As shown in the analytical HPLC profiles (Figure 8(a, b)), fragment condensation at position 23 proceeded much slower compared to that at position 20. In both cases the reaction was incomplete indicating the difficulty of the specific region.

Attempted Stepwise Synthesis

In order to study if we could obtain Mdm2 RING finger by the linear solid-phase synthesis, the peptide chain was elongated by the stepwise mode starting from the 20–48 resin-bound peptide

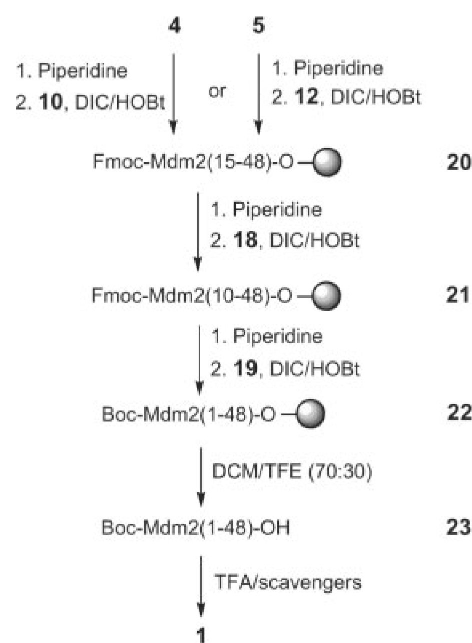

Figure 7. Four-fragment synthetic approach starting from the resin-bound 23–48 or 20–48 resin-bound peptides.

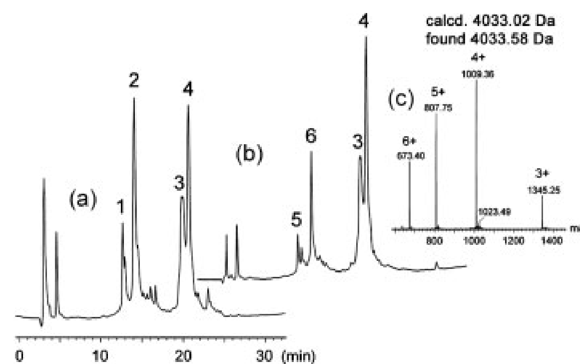

Figure 8. RP-HPLC analysis after side-chain deprotection of the mixtures obtained by the solid-phase condensation of (a) Fmoc-(15–22)-OH and H-(23–48)-O-CLTR, (b) Fmoc-(15–19)-OH and H-(20–48)-O-CLTR; conditions D. Peaks identified: 1 = Met[25](O)-(23–48), 2 = 23–48, 3 = Fmoc-Met[25](O)-(15–48), 4 = Fmoc-(15–48), 5 = Met[25](O)-(20–48), and 6 = 20–48. (c) ESI-MS of the produced Fmoc-(15–48) (peak 4).

5. The synthesis was continued without difficulties up to the His[18]-residue. Couplings following this position proceeded sluggishly and incompletely, particularly in the Cys[15]–His[18] region. Therefore, evaluation of the stepwise synthetic method was not continued.

Synthesis by Condensation in Solution

The synthetic studies described above indicated that the best suited fragment combinations to perform the condensation in solution could be those of the 1–22 (**25**) with 23–48, or the 1–19 fragment (**27**) with 20–48. Both *C*-fragments **25** and **27** contain glycine as the *C*-terminal amino acid which does not undergo racemization during its activation. The condensations at these positions on solid-phase proceeded with acceptable rates. Therefore, it was expected that their condensation in solution at the same points would be more efficient. Two possibilities for the fragment condensation in solution were studied. In the first

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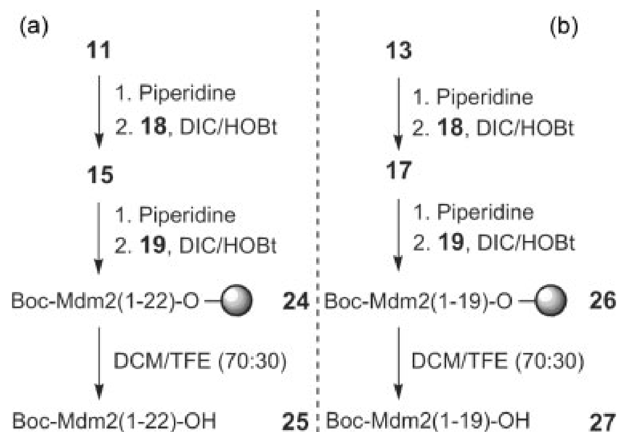

Figure 9. Synthesis of the Boc-(1–22) (a) and Boc-(1–19) (b) protected fragments.

case the 23–48 fragment was applied as its Clt ester, while in the second case the 20–48 fragment was applied with its carboxyl function unprotected.

The 1–22 and 1–19 protected peptides required for the fragment condensation in solution were prepared according to Figure 9(a, b), starting from the 15–22 and 15–19 resin-bound fragments. A twofold molar excess of 10–14 peptide **18** and 1–9 peptide **19** was correspondingly coupled onto resin-bound 15–22 and 15–19 peptides, respectively. In both cases peptides of high purity were obtained. Alternatively, fragments **24** and **26** were obtained by condensing peptide **19** with the 10–22 and 10–19 resin-bound fragments with similar results.

The required fragment 23–48 was quantitatively cleaved from the resin by treatment with TFE/DCM (30 : 70) for 2 h at RT. The resulting partially protected peptide **28** was then converted to its Clt ester (**29**) by reaction with a 1.5 M excess of Clt chloride and DIEA in DCM (Figure 10(a)). The esterification reaction was completed in 4 h at RT. The *N*-terminal Fmoc-group of **29** was subsequently removed by treatment with 2% DBU in NMP for 2 h at RT. The obtained 2-Clt ester **30** was dissolved in NMP and left to react with the 1–22 protected peptide **25** in a 1 : 1.1 molar ratio, using DIC/HOBt as condensing agent. The reaction was carried out for 6 h at RT. The protected 48mer 2-Clt ester **31** was precipitated by addition of chilled DEE. Before global deprotection, the 2-Clt protective group was removed by treatment with TFE/DCM (30 : 70) for 1.5 h at RT, in order to avoid its attachment onto thiol functions of the Cys residues, as described above. The content of the peptide **1** in the crude deprotected mixture was 23% (Figure 11(a)). After purification peptide **1** was obtained in 24% yield according to the starting *N*-component **30**.

The alternative approach for the condensation in solution was the application of the *N*-component with its carboxyl group unprotected (Figure 10(b)). This methodology offers the advantage of removing the *N*-Fmoc-group on the resin without the necessity of the esterification reaction. However, one must consider the reactivity of the free carboxyl group, which can participate in the condensation. To circumvent this problem a preactivation of the *C*-component was performed by treatment of the fragment **27** with equimolar amount of EDC/HOBt for 20 min at 4 °C and 20 min at RT. The *N*-component **32** was then added and the reaction was left to proceed for 2 h. After precipitation with water, whereby the formed EDC-urea and HOBt are removed, the obtained crude peptide **23** was deprotected, as above. The

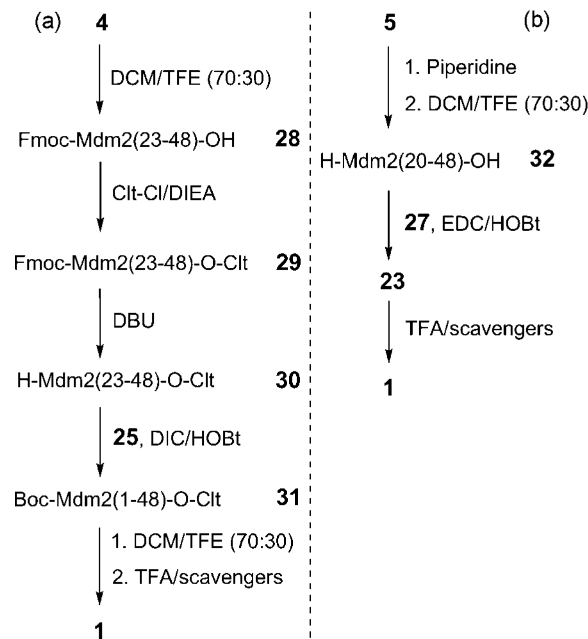

Figure 10. Synthesis of Mdm2 RING finger by the condensation in solution of (a) the 1–22 and 23–48 and (b) the 1–19 and 20–48 protected peptides.

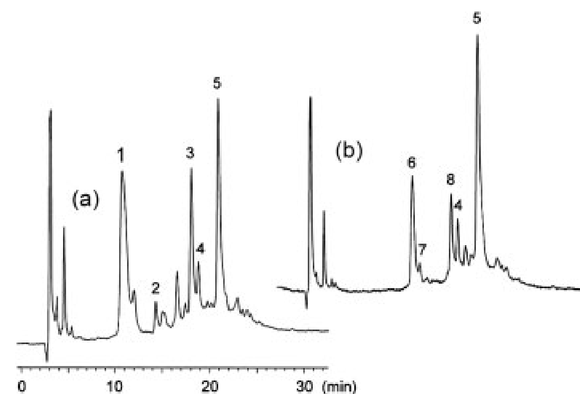

Figure 11. RP-HPLC analysis after global deprotection of the fragment condensation mixtures of (a) Boc-(1–22)-OH and H-(23–48)-O-Clt and (b) Boc-(1–19)-OH and H-(20–48)-OH in solution; conditions F. Peaks identified: 1 = 1–22, 2 = Met^{25}(O)-(23–48), 3 = 23–48, 4 = Met^{25}(O)-(1–48), 5 = 1–48, 6 = 1–19, 7 = Met^{25}(O)-(20–48), and 8 = 20–48.

content of peptide **1** in the crude deprotected mixture was 42% (Figure 11(b)). After purification peptide **1** was obtained in 37% yield, relative to the starting *N*-component **32**. In Figure 12 the RP-HPLC and the ESI-MS of the purified RING finger obtained from this synthetic method is shown.

Conclusions

In conclusion, the synthesis of the cysteine-rich Mdm2 RING finger domain was evaluated by employing fragment condensation on solid-phase and in solution. The latter method was performed using either protected or free peptides at the *C*-terminus as the amino component. The solution condensation method in which the *N*-component was applied with its *C*-terminal carboxyl group unprotected provided the best results. The developed method is well suited for larger scale synthesis of the Mdm2 RING finger

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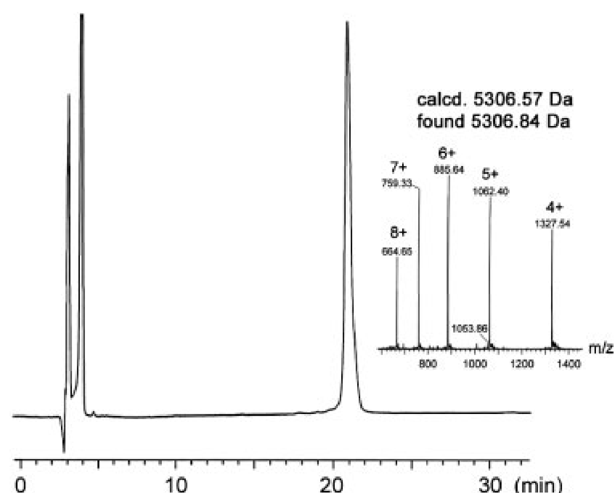

Figure 12. RP-HPLC analysis (conditions F) and ESI-MS of purified Mdm2 RING domain.

peptide and combines the advantages of both solid-phase and solution synthesis.

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Supporting information

Supporting information may be found in the online version of this article.

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