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Facile synthesis of C-terminal peptide hydrazide and thioester of NY-ESO-1 (A39-A68) from an Fmoc-hydrazine 2-chlorotrityl chloride resin

Yi-Chao Huang [a,b], Chen-Chen Chen [a], Si-Jian Li [a,c], Shuai Gao [b], Jing Shi [c,*], Yi-Ming Li [a,b,*]

[a] School of Medical Engineering, Hefei University of Technology, Hefei, Anhui 230009, China
[b] Department of Chemistry, Tsinghua University, Beijing 100084, China
[c] Department of Chemistry, University of Science and Technology of China, Hefei, Anhui 230026, China

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ABSTRACT

The NY-ESO-1 (A39-A68) peptide hydrazide was prepared through 9-fluorenyl-methoxycarbonyl solid-phase peptide synthesis (Fmoc SPPS) from a new 9-fluorenyl-methoxycarbonyl hydrazine 2-chlorotrityl chloride (Fmoc-hydrazine 2CTC) resin. The new resin was ideal for long-term storage and usage in Fmoc SPPS. Besides, the title peptide hydrazide could be transformed nearly quantitatively into the corresponding peptide thioester, which was both isolable and usable directly in native chemical ligation (NCL).

1. Introduction

NCL (native chemical ligation) has proved to be the most successful ligation method used in protein chemical synthesis.[1] This ligation method employs a chemoselective reaction between an N-terminal cysteine peptide and a C-terminal peptide thioester to generate the product with a native peptide bond.[2] Although the N-terminal cysteine peptide segment can be routinely prepared via both Boc (*tert*-butoxycarbonyl) and Fmoc (9-fluorenyl-methoxycarbonyl) based SPPS (solid-phase peptide synthesis), synthesis of the C-terminal peptide thioester requires a modified SPPS protocol.[3] Boc chemistry can attach the thioester moiety directly on resin via the 3-mercaptopropionamide linker.[4] However, the use of HF as the cleavage reagent to release the peptide thioester from the resin is potentially hazardous. On the other hand, Fmoc methodology uses a trifluoroacetic acid (TFA) as a much milder cleavage reagent, which is applicable to the acid-sensitive peptides that contain glycosylation or phosphorylation modifications. Unfortunately, thioesters are sensitive to iterative piperidine treatments needed during the Fmoc SPPS. Therefore, several strategies have been developed to circumvent this problem via modified Fmoc SPPS.[3] One group of strategies relies on the intramolecular *O*-to-*S* or *N*-to-*S* acyl transfer reactions,[5,6] whereas another group of methods involve the use of activating reagents to activate the peptide C-terminal group mediating its nucleophilic substitution by an external thiol.[7]

Regarding the second group of approaches, C-terminal peptide thioesters can be prepared via coupling with a thiol after release of the fully protected peptide acid (Fig. 1A),[8] thiolytic cleavage from a sulfonamide safety-catch linker (Fig. 1B),[9] or thiolytic cleavage of an *N*-acyl benzimidazolinone (Nbz) terminated peptide (Fig. 1C).[10] Recently, Harris and Brimble made a direct comparison of above three Fmoc methods and the conventional Boc chemistry for the generation of a model C-terminal peptide thioester NY-ESO-1 (C39-A68) (Fig. 1E).[11] The peptide segment belongs to the cancer protein NY-ESO-1, which requires chemical synthesis to rival recombinant expression for the development of cancer vaccines.[12] Harris and Brimble have reported that this peptide thioester could be synthesized via direct Boc SPPS or through thioesterification of a fully protected peptide acid after Fmoc chain-assembly. However, the Fmoc protocol was restricted to a relatively small batch scale cleavage (less than 0.04 mM) so that a more readily scalable Fmoc approach is needed. Surprisingly, both the safety-catch linker and

* Corresponding authors. Tel.: +86 551 62877080; e-mail address: lym2007@mail.ustc.edu.cn (Y.-M. Li).

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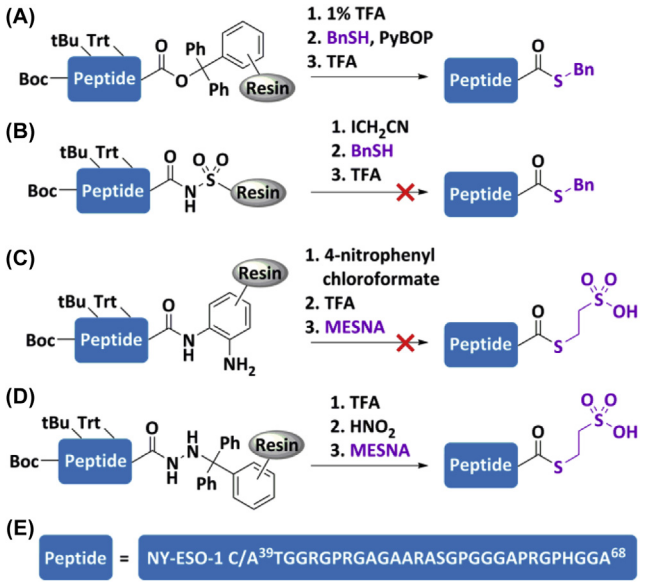

Fig. 1. Established methods of Fmoc-based thioester synthesis through an external activating reagent include thioesterification of (A) fully protected peptide acid released from the resin, (B) activated peptide C-terminal safe-catch linker, (C) peptide acyl benzimidazolinone, (D) oxidized peptide hydrazide. (E) Amino acid sequence of the cancer protein NY-ESO-1 Cys/Ala39-Ala68.

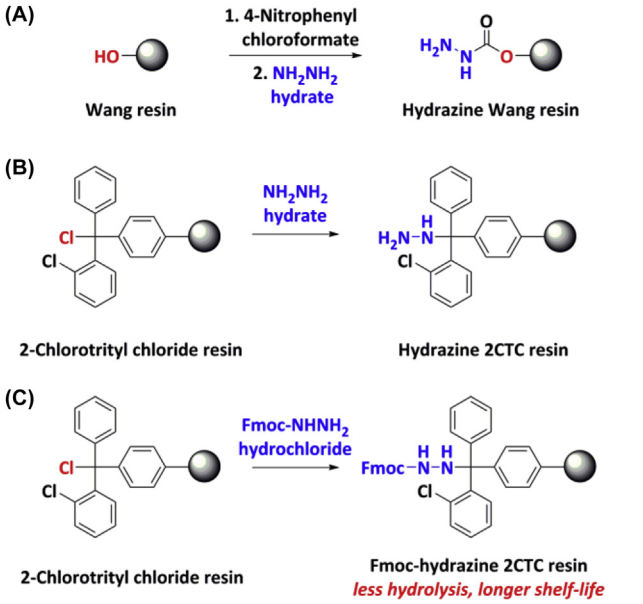

Fig. 2. Synthesis of hydrazine resins from commercially available resins (first generation hydrazine Wang resin, second generation hydrazine 2CTC resin, third-generation Fmoc-hydrazine 2CTC resin).

Nbz strategies failed to afford any detectable or separable peptide thioester products.

In 2011, peptide hydrazides were reported to be a thioester equivalent reagent in NCL.[13a] C-terminal peptide hydrazides undergo chemoselective oxidation and thioesterification to produce C-terminal peptide thioesters, which can be either isolated or directly used in NCL in situ in a one-pot manner.[13] We were interested to examine whether the hydrazide method[14] can be used to overcome the challenge of making the thioester of NY-ESO-1 (C39-A68) (Fig. 1D). Here, we describe the synthesis of NY-ESO-1 (A39-A68) C-terminal MESNA (sodium 2-sulfanylethanesulfonate) thioester through nearly quantitative oxidation and thioesterification of its hydrazide precursor in an isolated yield of 28% calculated from resin loading. Note that a single N-terminal amino acid mutation C39A was employed to prevent cyclization or oligomerization in the following NCL test. Employing one-pot thioesterification and ligation, we also synthesized NY-ESO-1 (A39-A68) C-terminal MPAA (4-mercaptophenylacetic acid) thioester, which was reacted directly in NCL to produce NY-ESO-1 (A39-G76) efficiently (HPLC yield 97%, isolated yield 70%). Furthermore, we demonstrated the use of a new Fmoc-hydrazine 2-chlorotrityl chloride (2-CTC) resin for the preparation of protein hydrazides. This resin was proven to give a higher substitution level from the starting 2-CTC resin compared with two earlier reported hydrazide-yielding resins.[13a,15] Importantly, Fmoc protection form of this new hydrazine resin extends its shelf life to at least 3 months at 4 °C, which is reliable for long-term storage and usage.

2. Results and discussion

To realize the abovementioned proposal, we required a C-terminal peptide hydrazide of NY-ESO-1 (A39-A68) for the following thioesterification and NCL reaction. One type of resins for the preparation of peptide hydrazides was derived from the Wang resin by treatment with 4-nitrophenyl chloroformate and hydrazine hydrate stepwise (Fig. 2A).[13a] Another approach to produce a hydrazine resin was reported by Barlos et al. (Fig. 2B).[15] This simple hydrazine 2-chlorotrityl chloride (2CTC) resin was proved to improve yields and purities of peptide hydrazides.[13b] However, the hydrazine moiety of this resin is unprotected so it has to be freshly prepared before Fmoc SPPS because of its sensitivity to moisture and air. Besides, treatment of hydrazine hydrate usually lowered the loading capacity of 2CTC owing to the incidental hydrolysis. Although anhydrous hydrazine might replace hydrazine hydrate to suppress the hydrolysis, this reagent is potentially explosive and dangerous to handle.[15] To overcome these drawbacks, we prepared a third-generation hydrazine resin termed Fmoc-hydrazine 2CTC resin (Fig. 2C). The starting 2CTC resin was first swollen in CH_2Cl_2 at 0 °C and then reacted with Fmoc hydrazine hydrochloride (4 equiv) and *N*,*N*-diisopropylethylamine (10 equiv) in DMF (*N*,*N*-dimethylformamide)/CH_2Cl_2 (1/1) at room temperature (Fig. S1). After reaction overnight, the active chloride was capped with methanol and the resulting resin was washed extensively with DMF, water, DMF, methanol and ethylether. Drying under high vacuum afforded the light-yellow powdered Fmoc-hydrazine 2CTC resin (Fig. S2), which could be stored for months at 4 °C. Next we compared the loading of our new resin with that of the hydrazine 2CTC resin prepared according to the previous literature[13b] by measuring UV absorptions at 301 nm of their Fmoc deprotection solutions. For the hydrazine 2CTC resin, the piperidine solution was collected after the standard HBTU coupling of Fmoc-Ala-OH. The loading of Fmoc-hydrazine 2CTC resin (0.39 mmol/g) was determined to be slightly higher than hydrazine 2CTC resin (0.34 mmol/g) prepared from the same batch 2CTC resin (0.57 mmol/g). In order to establish the robustness of our new resin, we used it to synthesize a model pentapeptide LYRAA C-terminal hydrazide via standard Fmoc SPPS (Fig. S3). Two peptide samples were obtained using freshly-made and three-month old Fmoc-hydrazine 2CTC resins, which showed almost identical HPLC and ESI-MS profiles (data not shown). The above experiments demonstrated that our new resin is competent for facile and practical Fmoc SPPS of peptide hydrazides.

With the third-generation Fmoc-hydrazine 2CTC resin in hand, we firstly synthesized the NY-ESO-1 (A39-A68) hydrazide at 0.1 mmol scales using the manual standard HBTU/Fmoc SPPS (Fig. 3). The new resin worked fairly well and gave the target peptide hydrazide in an isolated yield of 43% and >98% purity. Next we carried out the synthesis of the NY-ESO-1 (A39-A68) MESNA thioester to test the effectiveness of the hydrazide method (Fig. 4A). First, the starting hydrazide (1.5 mM) was dissolved in the activation

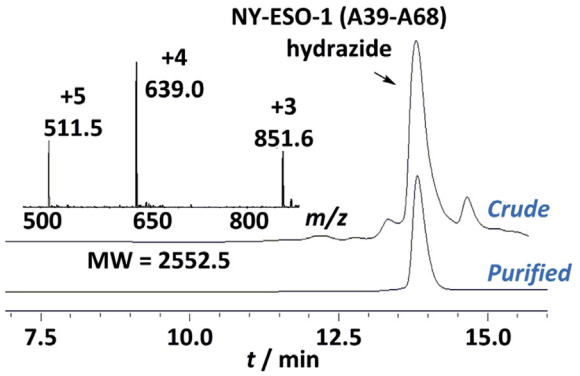

Fig. 3. Analytical HPLC trace and ESI-MS (obs: 2552.5; calcd: 2552.7) of the NY-ESO-1 (A39-A68) hydrazide.

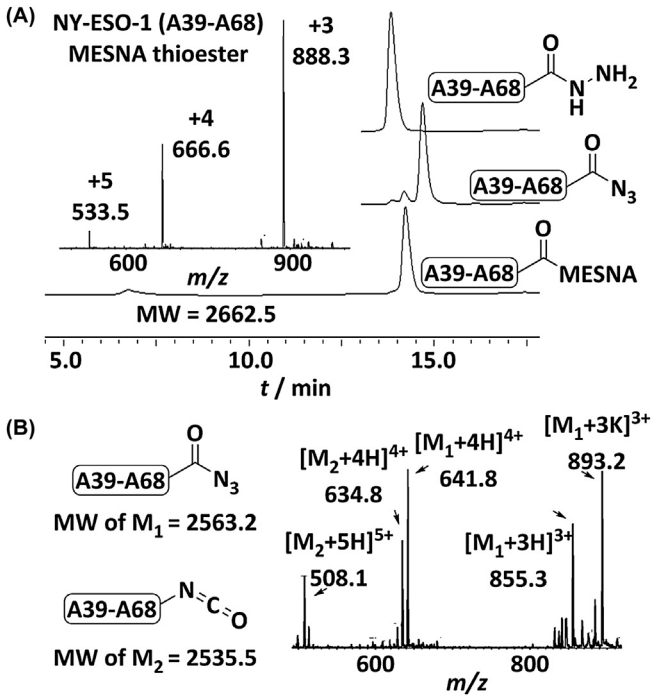

Fig. 4. (A) Analytical HPLC traces for the MESNA thioesterification of the NY-ESO-1 (A39-A68) hydrazide and ESI-MS (obs: 2662.5; calcd: 2662.9) of NY-ESO-1 (A39-A68) MESNA thioester. (B) ESI-MS characterization of the reaction intermediate NY-ESO-1 (A39-A68) acyl azide, for peptide acyl azide M_1 (obs: 2563.2; calcd: 2563.6), for peptide isocyanate after Curtius rearrangement M_2 (obs: 2535.5; calcd: 2535.7).

buffer (6.0 M Guanidinium chloride, 0.2 M NaH_2PO_4) and the reaction solution was kept at pH 3.0 and −10 °C. The oxidation started after addition of $NaNO_2$ (15 mM). After 30 min, analytical HPLC and ESI-MS confirmed the formation of the desired peptide C-terminal acyl azide intermediate. Interestingly, both the peptide acyl azide and peptide isocyanate were observed in the mass spectrum (Fig. 4B). The compound peptide isocyanate was probably generated through Curtius rearrangement from the peptide acyl azide in the mass spectrometer instead of in the reaction mixture. We thought that the rearrangement process should be significantly suppressed at lower pH and temperature. After 30 min hydrazide activation, MESNA was added in one portion and the reaction buffer was adjusted to pH 7.0. The thioesterification was kept for 10 min and monitored again through analytical HPLC and ESI-MS. Both experiments confirmed nearly quantitative conversion to the peptide MENSA thioester (Fig. 4A). The obtained thioester was immediately purified with semi-preparative HPLC (isolated yield 65% from the peptide hydrazide, 28% from the resin loading). Therefore, we gained solid access to NY-ESO-1 (A39-A68) thioester by employing the new Fmoc-hydrazine 2CTC resin and the well-defined hydrazide-to-thioester transformation protocol.

Next we planned to perform the peptide hydrazide-to-thioester transformation and native chemical ligation in a one-pot fashion. To accelerate NCL, we switched the thiol from MESNA to MPAA[16] because this thiol additive and its derivatives[17] have been demonstrated to be superior catalysts for NCL according to recent literature. NY-ESO-1 (A39-A68) MPAA thioester (1.5 mM) was obtained through the similar protocol as MESNA thioester and then ligated with NY-ESO-1 (C69-G74) (1.3 mg, 2.5 mM) in the ligation buffer (6.0 M Guanidinium chloride, 0.2 M NaH_2PO_4, 50 mM MPAA) at pH 7.0. The reaction finished within 60 min according to the clean peak-to-peak conversion shown in the analytical HPLC trace (Fig. 5A). The ligated product NY-ESO-1 (A39-G74) was immediately purified with semi-preparative HPLC and lyophilized (HPLC yield 97%, isolated yield 48%, 2.2 mg). The in situ formed MPAA thioester and ligation product were both confirmed by ESI-MS (Fig. 5B).

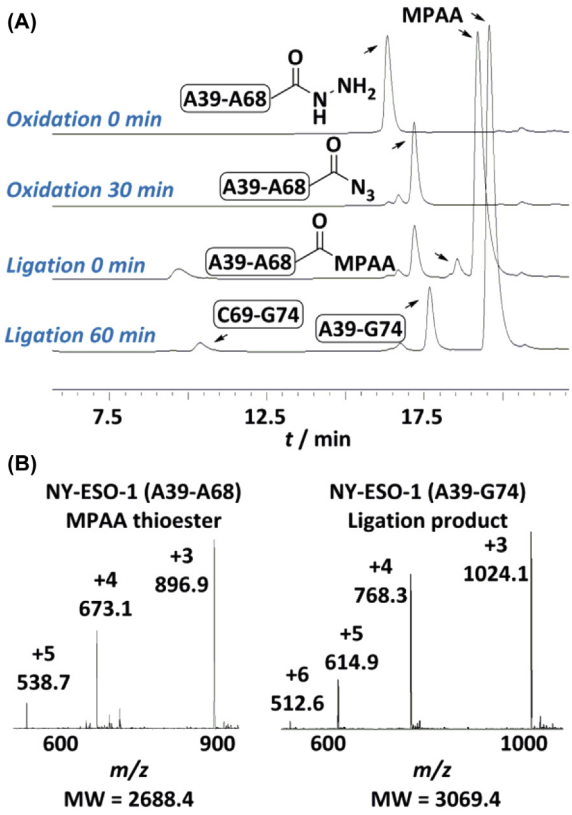

Fig. 5. (A) Analytical HPLC traces for the one-pot activation, thioesterification and ligation of the NY-ESO-1 (A39-A68) C-terminal hydrazide. (B) ESI-MS characterization of the reaction intermediate NY-ESO-1 (A39-A68) MPAA thioester (obs: 2688.4; calcd: 2688.9) and ligation product NY-ESO-1 (A39-G74) (obs: 3069.4; calcd: 3069.8).

Finally, to test whether the one-pot ligation protocol can be further streamlined, we did an experiment by premixing the peptide segments NY-ESO-1 (A39-A68) hydrazide with NY-ESO-1 (C69-G74) in the activation buffer. The following activation, thioesterification and ligation steps were similar as aforementioned. The reaction proved to proceed as efficiently as previously described (Fig. 6A). Interestingly, we observed the appearance and disappearance of S-nitroso[18] NY-ESO-1 (C69-G74) peptide upon oxidation and ligation, respectively (Fig. 6B). This could be explained by nitrosonium ion addition to the cysteine thiol group during oxidation. After the addition of MPAA into the reaction mixture, this process was reversed and NY-ESO-1 (C69-G74) was released to

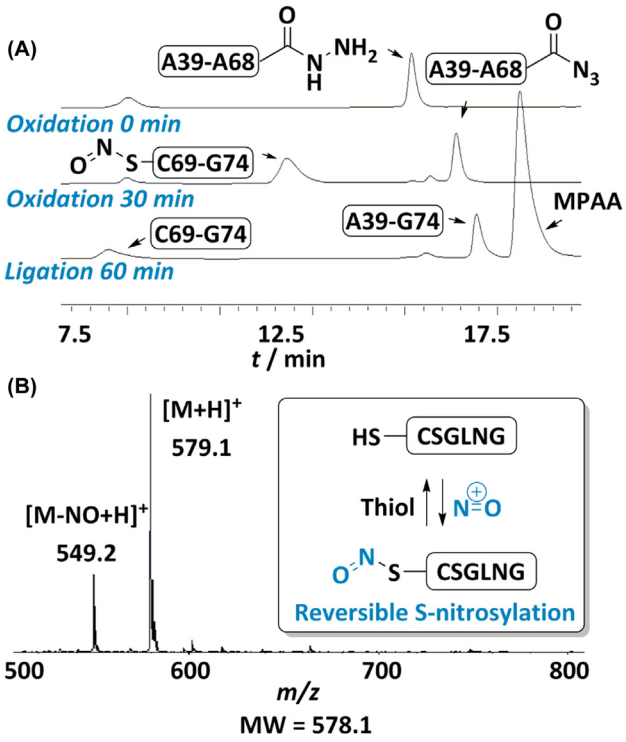

Fig. 6. (A) Analytical HPLC traces for the simplified one-pot activation, thioesterification and ligation of the NY-ESO-1 (A39-A68) C-terminal hydrazide. (B) ESI-MS characterization of the reaction intermediate S-nitroso NY-ESO-1 (C69-G74) (obs: 578.1; calcd: 578.2).

participate in NCL reaction. Therefore, the presence of free thiol groups in peptide segments should not interfere the hydrazide oxidation step.

3. Conclusion

To summarize, NY-ESO-1 (A39-A68) C-terminal thioester presented a potentially tricky target molecule to prepare via Fmoc SPPS. To solve this problem, we attempted a facile two-step oxidation and thioesterification protocol with NY-ESO-1 (A39-A68) C-terminal hydrazide as the starting peptide segment obtained from standard Fmoc SPPS. The thioester could be isolated as a stable MESNA alkyl thioester in a high yield. Moreover, the thioester could be used directly in NCL as a more active MPAA aryl thioester. Furthermore, we developed a new Fmoc-hydrazine 2CTC resin, which is suitable for long-term storage (several months at 4 °C) and usage for Fmoc SPPS of peptide hydrazides. We considered that the new resin would be a useful solid-phase anchor to the community of synthetic peptide and protein chemistry.

4. Experimental

4.1. General information

4.1.1. Materials. Fmoc-protected amino acids were purchased from GL Biochem (Shanghai, China). Fmoc-hydrazine hydrochloride was purchased from Ouhe Chemicals (Beijing, China). All solvents were purchased from Sinopharm Chemical Reagent Co. Ltd., Alfa Aesar China Co. Ltd., J&K Chemical Co. Ltd. All of the analytical-grade and HPLC-grade solvents were used as received without further purification.

4.1.2. HPLC. Analytical HPLC was run on an SHIMADZU (Prominence LC-20AT) instrument using an analytical column (Grace Vydac 'Peptide C18', 150×4.6 mm, 5 μm particle size, flow rate 1.2 mL/min, rt). Analytical injections were monitored at 214 nm and 254 nm. Semi-preparative HPLC was run on a SHIMADZU (Prominence LC-20AT) instrument using a semi-preparative column (Grace Vydac 'Peptide C18', 250×10 mm, 10 μm particle size, flow rate 4.0 mL/min). Solvent A was 0.1% TFA in acetonitrile, and solvent B was 0.1% TFA in water. Both solvents were filtered through 0.22 μm filter paper and sonicated for 20 min before use.

4.1.3. Fmoc SPPS. Screw-cap glass peptide synthesis reaction vessels were attained from commercial sources. 2CTC and Fmoc hydrazine 2CTC resins were initially swelled in DCM/DMF (1/1) for 10 min before use. Capping reagent was acetic anhydride/DIEA/DMF (1:1:8) (2×5 min). The resin was washed with 5×DMF, 5×DCM and 5×DMF. Deprotection reagent was 20% piperidine/DMF (5 min+10 min). The resin was washed with 5×DMF, 5×DCM and 5×DMF. The coupling step was carried out at 25 °C by pouring a preactivated solution of 4 equiv protected amino acid (0.4 M in DMF) with 3.6 equiv HBTU, 8 equiv of DIEA to the resin. For the single coupling, after 45 min, the resin was washed with 5×DMF, 5×DCM and 5×DMF. For the double-coupling, the reaction time was changed to 30 min and twice. The coupling efficiency was checked with qualitative chloranil test. Prepare 4% chloranil (w/v) DMF solution stored at 4 °C. Transfer a few resin beads to a 1.5 mL EP tube and add two drops of the chloranil reagent. Mix well and leave at room temperature for 3 min. Dark red beads indicate positive results while colorless or light yellow beads indicate negative results. Cleavage reagent was chosen as Reagent B. Typically a TFA cocktail of TFA/phenol/water/TIPS (88/5/5/2) was added to the dry resin prewashed with DCM. After 2–3 h, the resin was washed with an equal volume of TFA once. Combined elutents were concentrated by nitrogen blow. The crude peptides were obtained through precipitation with cold ether and centrifugation at 5000 rpm for 2 min. The peptide pellet was dissolved in 0.1% TFA containing CH_3CN/H_2O (1/1), characterized by analytical HPLC and ESI-MS, and purified by semi-preparative HPLC and lyophilized immediately.

4.1.4. Mass spectrometry. MALDI-TOF mass spectra were measured on an Applied Biosystems 4700 Proteomics Analyzer 283. A solution of 10 mg/ml matrix α-cyano-4-hydroxy cinnamic acid containing 1:1 v/v (0.1% TFA in acetonitrile/0.1% TFA in water) was used for generating the probe-matrix mixture. High-resolution ESI mass spectra were measured on Agilent 6210 Time of Flight Mass Spectrometer. Normal ESI mass spectra were measured on a Bruker Daltonics Data Analysis 3.0 workstation.

4.2. Synthesis of Fmoc hydrazine 2CTC resin

2-Chlorotrityl chloride resin (loading=0.57 mmol/g) (2 g) was swollen in CH_2Cl_2 (16 mL) at 0 °C. Fmoc hydrazine hydrochloride (1.16 g, 4 equiv) and diisopropylethylamine (2.02 mL, 10 equiv) were dissolved in DMF (20 mL) and CH_2Cl_2 (4 mL). The above solution was then added dropwise into the resin slurry at 0 °C. The reaction mixture was gently stirred from 0 °C to room temperature overnight (Fig. 7). Methanol (0.32 mL) was added to quench the remaining 2CTC resin. The resulting resin was washed with DMF 3×5 mL, H_2O 3×5 mL, DMF 3×5 mL, methanol 3×5 mL, ethylether

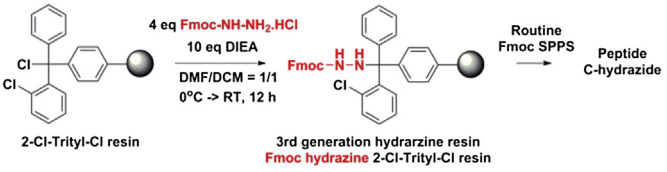

Fig. 7. The synthesis of third-generation hydrazine resin.

3×5 mL and kept under high vacuum for 2 h. The dried Fmoc hydrazine 2CTC resin was purged with nitrogen and stored at 4 °C.

4.3. Synthesis of NY-ESO-1 (A39-A68) hydrazide

Single-coupling/HBTU Fmoc SPPS of NY-ESO-1 (A39-A68) hydrazide was carried out from the Fmoc hydrazine 2CTC resin (0.39 mmol/g) as mentioned above. The synthetic scale was 0.1 mmol (256 mg resin). The target peptide was purified with semi-preparative HPLC and lyophillized (109 mg, isolated yield 43%).

4.4. Synthesis of NY-ESO-1 (C69-G74)

Single-coupling/HBTU Fmoc SPPS of NY-ESO-1 (C69-G74) was carried out from the 2CTC resin (0.57 mmol/g) as mentioned above. The synthetic scale was 0.1 mmol (172 mg resin). The target peptide was purified with semi-preparative HPLC and lyophillized (32 mg, isolated yield 59%).

4.5. Synthesis of NY-ESO-1 (A39-A68) MESNA thioester

NY-ESO-1 (A39-A68) hydrazide (3.8 mg, 1.5 mM) was dissolved in the activation buffer (0.8 mL) (6.0 M Guanidinium chloride, 0.2 M NaH_2PO_4). The reaction solution was kept at pH 3.0 and −10 °C. $NaNO_2$ (80 μL, 150 mM, final concentration 15 mM) was added. After 30 min, MESNA (5 mg, 30 mM) was added and pH was adjusted to 7.0. The thioesterification reaction was kept for 10 min at room temperature. NY-ESO-1 (A39-A68) MESNA thioester was immediately purified with semi-preparative HPLC and lyophillized (2.5 mg, isolated yield 65%).

4.6. Synthesis of NY-ESO-1 (A39-G74)

NY-ESO-1 (A39-A68) hydrazide (3.8 mg, 1.5 mM) was dissolved in the activation buffer (0.8 mL) (6.0 M Guanidinium chloride, 0.2 M NaH_2PO_4). The reaction solution was kept at pH 3.0 and −10 °C. $NaNO_2$ (80 μL, 150 mM, final concentration 15 mM) was added. After 30 min, MPAA (5 mg, 30 mM) and NY-ESO-1 (C69-G74) (1.3 mg, 2.5 mM) were added and pH was adjusted to 7.0. The ligation was kept for 60 min at room temperature. NY-ESO-1 (A39-G74) was immediately purified with semi-preparative HPLC and lyophillized (2.2 mg, isolated yield 48%).

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Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tet.2014.03.022.

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