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Anhydrous hydrogen fluoride cleavage in Boc solid phase peptide synthesis

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Abstract

Solid phase peptide synthesis using *tert*-butyloxycarbonyl/benzyl chemistry (Boc-SPPS) is important for producing peptides for fundamental research as well as for clinical use. During Boc-SPPS, liquid anhydrous hydrogen fluoride (HF) is used to remove the sidechain protecting groups of the assembled peptide and to release it from the resin. Here, we provide a detailed protocol for ‘HF cleavage’, aiming to improve accessibility and use of this valuable and well-validated technique.

Keywords

Solid phase peptide synthesis (SPPS); Boc chemistry; hydrogen fluoride (HF) cleavage

1 Introduction

Two key techniques in peptide synthesis are *tert*-butyloxycarbonyl (Boc)- and 9-Fluorenylmethoxycarbonyl (Fmoc)-solid phase peptide synthesis (SPPS) [1,2], providing peptides to the pharmaceutical industry [3] and to scientists working in basic research [4]. Fmoc-SPPS [5] is most commonly used, due to its overall safer handling methods and simpler cleavage setup. It is also preferred for peptides containing acid-sensitive modifications such as O-glycosidic or phosphate residues. However, many laboratories continue to use the original strategy, conceived by Merrifield in 1963[6–8], that takes advantage of differential labilities of Boc and benzyl (Bzl) sidechain-protecting groups towards acid. This Boc-SPPS strategy offers reliable synthesis of long polypeptides, alternative orthogonality regarding protecting groups, and easy production of C-terminal thioesters for native chemical ligation (NCL) [9,10]. Boc-SPPS is also used to synthesize ‘difficult’ peptides, which suffer from incomplete couplings and/or deprotection during Fmoc-SPPS [11–13].

Here, we present a protocol for HF cleavage, an integral part of Boc-SPPS, whereby liquid anhydrous HF removes the sidechain protecting groups of the assembled peptide and releases the peptide from the resin [14]. Anhydrous HF is a highly volatile, non-oxidizing acid with strong protonating ability, thus making it an excellent medium for dissolving peptides (see Box 2 in ref. [15] for details of the physicochemical properties of anhydrous

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HF). The highly corrosive nature of HF, which also reacts with silicon oxides to dissolve glass, requires a HF-resistant apparatus made from Teflon and Kel-F. HF efficiently removes the most common protecting groups used for Boc-SPPS *via* a S_N1 mechanism. This creates benzyl carbocations that need to be trapped by scavengers to prevent side reactions with amino acids with nucleophilic side chains [16]. Upon deprotection and cleavage from the resin, HF is evaporated and neutralized, and the crude peptide precipitated and prepared for purification.

The protocol is divided into three main parts including pre-cleavage sample preparation (30 min – 2.5 h), HF cleavage procedure (3 h) and reaction workup (30 min), which are briefly described here, followed by the comprehensive protocol in the later sections.

- (1) The pre-cleavage sample preparation starts with the peptide assembled by Boc-SPPS on resin using common protecting groups (see Table 1). Some amino acid sidechain protecting groups used in SPPS (e.g., His(Dnp) and Trp(CHO)) are not deprotected by HF. Therefore, deprotection of His(Dnp), removal of the N-terminal Boc group, and deprotection of Trp(CHO), if present, are carried out in this order before HF treatment, to avoid side reactions such as alkylation (e.g., *tert*-butylation of sensitive residues). The peptide-resin is then dried under N_2 flow and transferred to the HF cleavage reaction vessels in which scavengers are added.
- (2) The HF cleavage procedure (Figure 1) starts with transfer of HF from the HF cylinder into the first collection vessel (CV1), which is cooled by a solvent/dry-ice bath. From CV1, HF is then transferred into the smaller reaction vessels (RV1, RV2), which contain the peptide-resin-scavenger mixture, using a temperature gradient under reduced pressure. The cleavage reaction occurs in the reaction vessels for 1 h at $-5\text{ }^{\circ}\text{C}$ to $0\text{ }^{\circ}\text{C}$. HF is then transferred under vacuum to a calcium oxide (CaO) trap for neutralization (*see* Box 1).
- (3) The reaction workup starts once all HF has evaporated and the reaction vessels have been taken off. The peptide is precipitated with cold ether, washed with more cold ether to remove all scavengers, and then dissolved with a peptide solubilizing solution and lyophilized.

In the following sections, we provide the comprehensive details required to execute this HF cleavage protocol. This includes apparatus setup, reagent preparation and safe handling of anhydrous HF, with the aim of encouraging safe use of this reliable, valuable and effective technique.

2 Materials

CAUTION: All chemicals used in this protocol are potentially harmful; thus, a laboratory coat, gloves and eye protection are mandatory. Use deionized water to prepare reagents. Some of the listed material might also be available from other suppliers; we have listed the companies solely based on what we have used in the past.

2.1 Pre-cleavage sample preparation

1. Peptide assembled by Boc-SPPS (see [12,17,15]; 0.05–0.5-mmol scale; *see* Note 1) on resin in *N,N*-dimethylformamide (DMF; peptide synthesis grade) using Boc-amino acids containing common protecting groups (*see* Table 1)
2. SPPS vessel
3. Vacuum pump (Vacuubrand)
4. Dnp deprotection solution (*see* Note 2): 50 mL of 20% 2-mercaptoethanol (*see* Note 3) and 10% *N,N*-diisopropylethylamine (DIEA) in DMF (vol/vol)
5. Spatula
6. Trifluoroacetic acid (TFA; *see* Note 4)
7. Acid neutralization solution: 50 mL of 10% *N,N*-diisopropylethylamine (DIEA) in DMF (vol/vol)
8. Deformylation solution (*see* Note 5): Prepare 50 mL of 6% (vol/vol; 3 mL) ethanolamine (aminoethanol) and 5% (vol/vol; 2.5 mL) water in DMF
9. Dichloromethane
10. Methanol (MeOH)
11. N₂ gas line, hose with funnel to dry the resin
12. Mass balance

2.2 HF cleavage procedure

1. Safety equipment: apron (McMaster-Carr), TFA-resistant gloves (Scientific Shield), HF-resistant elbow-long gloves (preferably neoprene gloves; do not use nitrile gloves) (Fisher Scientific), safety glasses, laboratory coat, safety shower, dedicated HF fume hood, emergency eye rinse and HF first aid
2. HF reaction apparatus (Peptide Institute; *see* Note 6) with detailed setup instructions from manufacturer. It should come with vacuum gauge (*see* Note 7), connector and holder.
3. 3× Dewars to cool down reaction and collection vessels
4. 1× large Dewar to prepare dry-ice mixtures
5. Dry ice and 1× container for dry ice
6. Isopropanol (*i*-PrOH)
7. 3× 1-L beakers for water baths
8. Thermometer, range –20 to 100 °C
9. Reaction vessels with fitting caps and magnetic stir bars
10. Anhydrous hydrogen fluoride (HF, Matheson Tri-Gas, Praxair or GHC Gerling, Holz & Co. Handels; *see* Note 8) CAUTION: HF is a dangerous liquid (*see* Note

- 9). Do not use glassware to contain HF, as HF reacts rapidly and exothermically with glass.
11. HF-specific first aid kit (*see* Note 9).
 12. Flashlight to facilitate volume determination in collection and reaction vessels and to check for HF or blockages in tubes
 13. Wire or tweezers to remove the caps of the reaction vessels
 14. Water-ice-salt mixtures: prepare a 500-mL beaker with 70% ice, 30% water and a few scoops of NaCl (~70 g). Stir until it becomes a water-ice-salt slurry.
 15. Timer
 16. For CaO trap: HF adsorption cylinder (Peptide Institute; PeptaNova; CS Bio), CaO (CaO coarse, ~1 cm diameter, CS Bio; CaO fine, Sigma-Aldrich), dust mask, Teflon shavings (Peptide Institute; CS Bio), sieve for coarse CaO pellets, Fluorogrease (Peptide Institute).
 17. Log book to record HF usage for CaO trap, maintenance and comments
 18. Scavengers (*see* Note 10): anisole (methoxybenzene); 1,4-butanedithiol; *p*-cresol (*see* Note 11); *p*-thiocresol (4-methylbenzenethiol; *see* Note 12); 1,2-ethanedithiol; methoxyamine hydrochloride.
 19. 2× medium magnetic stir bars for CV1 and CV2
 20. 3× large magnetic stir bars for water baths
 21. 3× magnetic stir plates
 22. Filtration set-up
 23. Diethyl ether (*see* Note 13)
 24. 1× 500-mL beaker with water-ice mixture for each reaction vessel

2.3 Reaction workup

1. Extraction syringe/tube with filter (Scharlau: empty SPE tube 60 mL with 2 frits ExtraBond; or Alltech/Fisher: SPE columns, extract-clean empty reservoirs, polypropylene, 75 mL, plus 20 µm PE frits)
2. Lyophilizer (check pump specifications for HF compatibility as residual HF will be present in the crude peptide solution)
3. Filtration and lyophilization vessel (e.g., Falcon tubes, round bottoms, Erlenmeyer)
4. Dedicated plastic bottle for diethyl ether and scavenger waste
5. Analytical reversed-phase high-performance liquid chromatography (RP-HPLC) system (Agilent 1100 Series) with a suitable analytical column (C₁₈/C₈/C₄)

6. Liquid chromatograph coupled to a mass spectrometer (LC-MS; Waters Micromass ZQ Mass Spectrometer with 2695/2795 HPLC Separations Module) with C₁₈ analytical column (Sunfire, C₁₈, 3.5 μ m, 4.6 $\times$ 100 mm)

3 Methods

We advise not cleaving all material at once, in case optimization of the scavengers and workup conditions is required. Tips for troubleshooting are given in the Notes, but we also refer the reader to Table 2 in ref. [15].

3.1 Pre-cleavage sample preparation

Deprotection and sample preparation before HF cleavage is ideally carried out in SPPS vessels [12]. Important note: on-resin Dnp and CHO removal is not compatible with thioester peptides. For advice on dealing with this, see Table 1. For peptides lacking His(Dnp), skip steps 1-5 and proceed directly to step 6 below. For peptides without Trp(CHO), skip steps 12-14.

3.1.1 Dnp removal (include these steps if peptide contains His(Dnp))

1. Flow-wash peptide-resin in the SPPS vessel with DMF for 30 s (see Note 14).
2. Add 15 mL of Dnp deprotection solution and leave for 30 min; stir occasionally with a spatula. This removes the Dnp group from histidine.
3. Drain the solution and flow-wash with DMF for 30 s
4. Repeat steps 2 and 3.
5. Drain DMF, flow-wash with dichloromethane for 15 s to prevent exothermic DMF-TFA reaction.

3.1.2 N-terminal Boc deprotection

6. Add 5 mL of TFA and leave for 1 min. This removes the *N*-terminal Boc group.
7. Drain TFA.
8. Add 5 mL of TFA and leave for 5 min.
9. Drain TFA and flow-wash with DMF for 1 min.
10. Add 10 mL of acid neutralization solution and leave for 1 min.
11. Drain and repeat step 10.

3.1.3 Deformylation of Trp (include these steps if peptide contains Trp(CHO))

12. Drain and add 10 mL of deformylation solution; let it react for 30 min and stir occasionally. This removes the formyl group of tryptophan.
13. Drain and flow-wash with DMF for 30 s.
14. Repeat steps 12 and 13.

3.1.4 Final resin washing and drying

15. Flow-wash with 50% (vol/vol) dichloromethane/MeOH for 1 min, then drain.
16. Dry the peptide-resin under vacuum and N₂ flow for 15 min.
17. Weigh the resin into the reaction vessel (*see* Note 15).

3.2 HF cleavage procedure

CAUTION: All procedures requiring HF must be performed in HF-resistant apparatus installed in a fume hood, preferably in a dedicated room with dedicated equipment. Appropriate personal protective equipment should be used at all times. For detailed advice for the safe handling of HF, as well as first aid measures, we refer the reader to Boxes 4 and 5 in ref. [15].

1. Prepare all the equipment required for HF cleavage (*see* Materials section)
2. Check that the CaO trap still has the capacity to neutralize the planned amount of HF– if not, empty and refill it (*see* Box 1).
3. With the HF cylinder and taps 3 & 4 fully closed, start the vacuum pump. Open taps 1, 2, 5 and 6, check vacuum gauge.
4. Prepare 500 mL of dry-ice/solvent (MeOH, EtOH or *i*-PrOH) mixture and fill the three Dewars.
5. Cool down CV1 with dry-ice/solvent Dewar for at least 15 min.
6. Prepare 700 mL of warm tap water (25–50 °C) in a 1-L beaker.
7. Add scavenger (Table 2) to the peptide-resin in the reaction vessels.
8. Freeze the scavenger-peptide-resin mixture by placing reaction vessels into dry-ice mixture for at least 5 min.
9. Add a magnetic stir bar on top of the frozen scavenger-peptide-resin mixture (*see* Note 16), close the reaction vessel with a cap (the cap ridge should connect with the O-ring of the HF apparatus) and attach it to the HF cleavage apparatus.
10. Evacuate the reaction vessels by opening taps 3 and 4 (Figure 1a). Cool the reaction vessels for at least 10 min using the dry-ice/solvent-Dewar raised from underneath the reaction vessel. [Figure 1 near here]
11. At this point, double-check the vacuum, the dry ice in the Dewars, the temperature of the water bath (25–50 °C) and the correct orientation of all taps.
12. Close tap 2 and open the HF cylinder slowly to distil HF into CV1 (*see* Note 17; Figure 1b). Lower the Dewar from CV1 temporarily to determine how much HF has been distilled (*see* Note 18).
13. **CLOSE HF CYLINDER** (ensure handle is turned in the correct direction): tap 2 should be closed, and the other taps should be open (*see* Note 19).
14. To heat CV1, replace the CV1 Dewar with the warm water bath on top of a magnetic stir plate and warm the HF in CV1 for a maximum of 12 min or until a

slight positive pressure has been reached at the vacuum gauge between the HF cylinder and tap 2 (this step takes ~15 min; *see* Note 20; Figure 1c)

15. To transfer HF from CV1 to the reaction vessels, close taps 3 and 4, then close tap 5, then open tap 2. Next, open taps 3 and 4 simultaneously (Figure 1d).
16. Transfer 9 mL of HF into each reaction vessel (or 18 mL for 1–2 g of resin) (*see* Note 21). Temporarily remove Dewars and use either the markings on the reaction vessel or a measuring stick to determine the volume of distilled HF (*see* Note 22) (Figure 1d). Once sufficient HF has been transferred, close taps 2, 3 and 4. Once they are closed open tap 5 (Figure 1e).
17. If further cleavages are planned, exchange the warm water bath with a freshly-made dry-ice/solvent Dewar to cool CV1 (Figure 1e). Otherwise, remove the warm water bath, keep CV1 stirring and open tap 2 to transfer the remaining HF to the CaO trap (this step takes ~10 min; *see* Note 23, Figure 1f).
18. For the cleavage reaction, replace the reaction vessel Dewars with the water-ice-salt mixtures (-5°C) (*see* Note 24; Figure 1e). The cleavage reaction should last for 1 h –start the timer once the peptide-resin-scavenger mixture thaws and becomes suspended in the HF (*see* Note 25).
19. After the cleavage is complete, gradually open taps 3 and 4 in clockwise direction (as indicated by the arrows on the taps) to evaporate HF into the CaO trap (Figure 1g). Do not allow the HF/peptide mixture to bubble over half the reaction vessel height (this step takes ~10 min; *see* Note 26). Once most of the HF is evaporated, turn taps 3 and 4 to the fully open position. Continue evaporation for 2 min after the last visible bubbling of HF is observed. The remaining liquid is a mixture of peptide, scavenger and traces of HF.
20. Prepare the filtration setup (Figure 2) and take cold diethyl ether from the freezer, keeping the bottle cooled with dry ice.
21. Take off the reaction vessels and place them into the 500-mL water-ice beaker on top of the magnetic stir plate.
22. Remove the caps from the reaction vessels (*see* Note 27) and put them in the water-ice beaker to dilute any traces of HF. Add 30 mL of cold diethyl ether to the reaction vessels to precipitate peptide. Ensure that the mixture being stirred while adding the ether. Leave stirring for 2 min; a white precipitate usually forms.
23. If no further cleavages are planned, ensure that all the HF has been transferred to the CaO trap (HF cylinder and taps 3 & 4 closed, all other taps open for ~10 min), vent the HF apparatus (~10 min) and turn the vacuum pump off.

3.3 Reaction workup

1. Filter the precipitate and wash it with another reaction vessel volume of cold diethyl ether (this step takes ~20 min; *see* Note 28). Dispose of diethyl ether in a

dedicated plastic waste bottle that is kept in a fumehood as the ether washings contain odorous scavengers and residual HF.

2. Place the lyophilization flask underneath the filter syringes. Add the peptide solubilizing solution into the reaction vessel to dissolve the peptide pellet, and then transfer the solution into the filter syringe to dissolve the rest of the peptide.
3. Freeze the peptide solution and put it on the lyophilizer. Once it is dry, run an analytical RP-HPLC and mass spectrometry to determine the purity and confirm the mass of the peptide. Purification is generally carried out *via* (semi-)preparative RP-HPLC, and peptides should be kept at -20°C for long-term storage.

4 Notes

1. Plan around one day for a 20-mer peptide.
2. Prepare this solution if His(Dnp) is present in the peptide. The solution should be prepared freshly.
3. CAUTION: 2-Mercaptoethanol has a strong irritating smell; handle it with care and leave everything in the fume hood; use bleach to neutralize gloves, pipette tips and glassware that came in contact with it.
4. CAUTION: TFA is a dangerous fuming acid that can penetrate nitrile gloves; wear a laboratory coat, protection glasses and TFA-resistant gloves.
5. Prepare this solution if Trp(CHO) is present in the peptide. The solution should be freshly prepared.
6. The HF apparatus is also sold through Peptides International in the US and PeptaNova in Europe. A similar apparatus is available from US manufacturer CS Bio. The procedure described here is for the simultaneous cleavage of two peptides using the type I HF cleavage apparatus from Peptide Institute.
7. The location of the vacuum gauge is not always the same: it can be found either between the HF cylinder and CV1, between CV1 and the CaO trap *via* an additional tube, or between CV2 and the CaO trap. An advantage of having the vacuum gauge between the HF cylinder and CV1 is that the expansion from cooled/liquid to heated/gas phase during the HF transfer phase can be directly monitored, providing an extra degree of control (Figure 1c).
8. CAUTION: Carefully read the label on the HF cylinder before attaching it to the HF apparatus. We recommend using HF cylinders without a full-length eductor tube (FLET) and without a N_2 pressure pad. HF cylinders with both a FLET tube and N_2 layer are designed for delivery of liquid HF under pressure and are NOT compatible with the HF apparatus described here. Some cylinders have a pressure layer of N_2 on top of the HF, which must be removed by pulling the N_2 through the HF apparatus and the CaO trap before the HF can be used. Ensure the cylinder is safely attached to the wall or fume hood using approved brackets,

chains or clamps. Ideally, the tube connecting the HF cylinder and the HF apparatus should also be secured with an additional clamp, since too much pressure (e.g., from a N₂ pressure pad) can cause this tube to pop out of the apparatus. Clearly label the opening and closing directions of the HF cylinder and add a label to remind users to close the HF cylinder after the transfer.

9. Anhydrous HF is a fuming colorless liquid or gas with a strong, irritating odor. It causes immediate burning and severe pain and a whitish discoloration of the skin that usually proceeds to blister formation [18,19]. Systemic adverse health effects can occur by exposure to HF through skin contact, inhalation or ingestion. Adverse effects include irritation of the skin, eyes, mucous membranes and respiratory tract; nausea and vomiting; accumulation of fluid in the lungs; gastric pain; tissue destruction and burns; irregular heart rate; low blood calcium; and even death. For details of protection equipment and first aid kit/measures see ref. [15].
10. The scavengers, particularly *p*-thiocresol, have a strong irritating smell; handle them in the fume hood; use bleach to neutralize gloves, pipette tips and glassware that came in contact with them.
11. Should be a colorless liquid; can be stored as 5-mL aliquots, wrapped in aluminum, in a freezer at –20 °C.
12. Can be stored as 5-mL aliquots, wrapped in aluminum, in a freezer at –20 °C.
13. High-quality peroxide-free diethyl ether (e.g., with butylated hydroxytoluene inhibitor) is recommended to avoid oxidation of sensitive residues.
14. Flow-wash is defined in this protocol as the continuous addition and removal of solvent to the SPPS vessel using vacuum suction (flow rate ~1–5 mL/s).
15. The reaction vessel can hold up to 2 g of resin. 50 mg – 1 g is cleaved with 9 mL HF + 1 mL scavenger, 1–2 g of resin with 18 mL HF and 2 mL scavenger. Typically, 200 mg – 1 g of resin is cleaved per reaction vessel.
16. Ensure that the mixture is frozen before adding the stir bar; otherwise, the stir bar can get stuck in the frozen mixture, and will not start stirring after HF transfer.
17. Use 10 mL HF per cleavage of 50 mg – 1 g resin; 20 mL for >1 g resin; plus an extra 10 mL to facilitate the transfer. Transfer can take between 10 s and 15 min, with the transfer rate depending on the amount of HF left in the cylinder and on whether HF is sourced from the bottom (FLET, liquid outlet, fast) or from the top (gooseneck tube, gas outlet, slow). Slow transfer may also be a result of tube blockages. Quickly opening and closing tap 2 may help speed up the transfer. Only transfer the amount of HF you need for the day. Do not leave HF in the collection vessel overnight, and do not perform cleavages outside normal working hours, at the weekend or when there are no other trained personnel present.
18. We recommend using a flashlight to help you visualize the HF level.

19. Not closing the HF cylinder properly can be extremely dangerous, as HF is continuously pulled into the CaO trap. Overloading the trap results in an exothermic reaction that may melt the trap lid, damaging the apparatus and potentially releasing HF. We recommend clearly marking the open/close direction of the HF cylinder.
20. The warm water may need to be replaced to maintain the temperature. Ensure that both stir bars (inside CV1 and water bath) are stirring.
21. Transfer can take between 1–15 min depending on the vacuum (should be below 20 mbar), the amount of HF in CV1, the temperature of the water bath (ensure that the reaction vessels and CV1 have been immersed fully in cold and warm baths at the correct temperatures) and tube blockages. Briefly opening and closing tap 5 to re-evacuate the reaction vessels might improve HF flow. Slow HF transfer indicates that the HF apparatus requires maintenance.
22. We recommend using a flashlight to back-light the reaction vessels.
23. To avoid rapid heating of the CaO trap, do not transfer more than 30 mL of HF at any given time. If more than 30 mL needs to be neutralized, transfer the HF in batches (30–40 mL/h with a 4-kg CaO trap), and monitor the temperature of the trap by touch.
24. Ensure that the reaction vessel stir bars are stirring and that the reaction vessels are fully immersed in the water-ice-salt mixtures.
25. The cleavage mixture often goes from yellow to red to purple as protecting groups are removed from the peptide and subsequently react with the scavenger (especially when cleaving MBHA resins). Ensure that the reaction temperature stays between -5 and 0°C . Removal rate is slow for Arg(Tos), Lys(ClZ), and Cys(Meb) (Tos, toluenesulfonyl; ClZ, 2-chlorobenzoyloxycarbonyl; Meb, 4-methyl-benzyl;) if the water-ice-salt bath is below -5°C – this can result in incomplete removal. The reaction time can also be increased to 1.5 h if protection group removal is incomplete.
26. The reaction temperature should be at -5°C during HF evaporation to avoid side reactions. The reaction mixture can ‘bump or jump’ if the taps are opened too quickly, thereby blocking or contaminating the tubes with resin. To avoid this, turn the taps gradually in a clockwise direction without fully opening them.
27. Take the caps off carefully using either tweezers or wire and pulling the caps away from your body. Wear protection gear, as HF fumes can remain in the reaction vessels.
28. If the filter clogs, we recommend using gentle positive N_2 pressure to speed filtration up or using a new filter syringe. Alternatively, cover the syringe top with a gloved hand; ether evaporation will produce positive pressure to speed up filtration. If the filtrate is white and cloudy (a sign that some peptide precipitate has passed through the filter), wait until the peptide precipitate forms a layer or

pellet on top of the filter, then filter the cloudy diethyl ether solution again until the filtrate is clear.

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Text for Box 1

We recommend preparing the CaO trap (Figure 3) with 4 kg of CaO. CaO reacts with HF to form calcium fluoride (CaF_2) and water, and some of the water generated can further react with CaO to form $\text{Ca}(\text{OH})_2$. We calculated that 1 kg of CaO can neutralize up to 361 mL of HF. Since HF released into the trap is unlikely to reach all the CaO present, we add another 25% safety margin, replacing our 4 kg of CaO whenever 1,000 mL of HF has been distilled into the trap.

Filling the trap: Using a dust mask, laboratory coat, gloves and safety glasses, fill one-fifth of the HF adsorption cylinder with Teflon shavings (Teflon layers result in better HF distribution/contact with the CaO and lower the chance of blockage), then one-fifth with coarse CaO pebbles (use sieve if necessary, to get pebbles of ~1 cm in diameter). Continue alternating Teflon and CaO layers up to 5 cm below the top of the cylinder (the last CaO layer can be smaller in size to maximize absorption).

Connecting the trap: Clean the O-ring area, apply the O-ring and seal the cylinder using the top and the four screws. Connect the cylinder in between the vacuum pump and the HF cleavage apparatus (the top line goes to the vacuum pump and the side line goes to the HF apparatus). Check the vacuum of the cleavage apparatus by completely evacuating the system then closing off the vacuum pump and monitoring for 1 h whether the vacuum is maintained. If necessary, apply a small amount of Fluorogrease to improve the seal.

Emptying the trap: Once the HF adsorption capacity of the cylinder has been reached, empty and refill the CaO trap. Record how much HF has been distilled into the CaO trap to keep track of when its capacity has been reached. Another indication for trap saturation is which part of the cylinder is heating up as a result of HF neutralization: traps freshly filled with CaO primarily warm up at the bottom, whereas a nearly saturated trap warms up towards the top. Leave the apparatus and the CaO trap overnight at atmospheric pressure before emptying the trap. Use a dust mask and HF-protecting gear to clean the trap in the fume hood. The $\text{CaF}_2/\text{CaO}/\text{Ca}(\text{OH})_2$ mixture can be disposed of similarly to SiO_2 waste.

Figure 1.

Scheme illustrating the key steps during a typical HF cleavage. The HF apparatus is equipped with a vacuum line on the left side, and it operates under the principle that HF can be condensed from a warmer collection vessel (CV) to a colder CV or reaction vessel (RV). Red arrows indicate the direction of HF transfer during each step; explanation of the other symbols is provided in the legend. **(a)** The HF cleavage protocol starts with the CV closest to the HF cylinder (CV1), well as RV1 and RV2, cooled (-78°C), and the entire apparatus under vacuum. **(b)** HF transfer from HF cylinder into cooled CV1. **(c)** HF in CV1 is heated

(25-50 °C) in a water bath for 12 min or until the gauge shows positive pressure. **(d)** HF transfer from CV1 to RV1 and RV2. **(e)** HF cleavage at −5 to 0 °C for 1 h. **(f)** Removal of excess HF from CV1 into CaO trap. **(g)** Removal of HF from RV1 and RV2 once reaction is complete (CV1 Dewar is required only if further cleavages are planned and HF is in CV1).

Figure 2.
Sample workup/filtration setup

Figure 3.

CaO trap showing inlet (HF apparatus) and outlet (vacuum pump) connections. Partial cross-section depicts alternating layers of Teflon shavings and coarse CaO pebbles (which mix to five layers in a 4kg CaO trap). The top CaO layer is composed of fine CaO pebbles.

Table 1
Commonly-used sidechain protecting groups for Boc-amino acids

Amino acid	Protecting group	Special cleavage conditions	Remarks
Arg	Tos		
Asp/Glu	OcHex OBzl		Preferred over OBzl
Asn/Gln	Xan		
Cys	Meb Acm *		
His	Bom Dnp *	Thiolysis (before HF)	Use MeONH ₂ .HCl as additional scavenger [20] Pre-cleavage Dnp removal not compatible with thioester. Dnp group can be removed before (use sodium 2-mercaptoethanesulfonate (MESNa), pH 7.5; will also convert to 2-sulfoethyl thioester) or during NCL
Lys	2ClZ		
Ser/Thr	Bzl		
Trp	CHO *	Ethanolamine/DMF (before HF) HF + thiols (EDT/BDT) [21]	Pre-cleavage removal not compatible with thioester peptides The use of <i>p</i> -thiocresol may result in partial CHO removal. CHO can be removed after NCL [22]
	Hoc		Recommended for thioester peptides
Tyr	2BrZ		

* HF-stable sidechain protecting groups

EDT = 1,2-ethanedithiol; BDT = 1,4-butanedithiol; NCL = native chemical ligation

Table 2
Cleavage cocktail (scavenger) recommendation. Selection of the proper scavenger cocktail is crucial for minimizing deleterious side reactions during HF cleavage.

Recommended use	Cleavage cocktails	Ratio
Standard peptides	HF:anisole	(9:1, v/v)
Multiple Glu/Asp	HF: <i>p</i> -cresol	(9:1, v/v)
Multiple Cys	HF: <i>p</i> -cresol: <i>p</i> -thiocresol	(18:1:1; v/v)
Thioester, Trp(CHO) and/or His(Bom)	HF:anisole:BDT: MeONH ₂ .HCl	(18:2:1:1; v/v/v/w)