Automated Peptide-Resin Deprotection/Cleavage by a Robotic Workstation

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INTRODUCTION

Automated multiple-peptide synthesis technology has revolutionized peptide research by increasing the rate and ease of peptide synthesis (5,6,8,12, 14). Although the synthesis of peptides has been automated, the subsequent side-chain deprotection and cleavage from the resin still require manual workup and can quickly become the rate-limiting steps in obtaining peptides.

Peptides synthesized by standard Merrifield solid-phase chemistry require side-chain deprotection and concomitant cleavage from the resin to obtain the free peptide (13). For peptides synthesized by Fmoc chemistry, this can be accomplished by treating the peptide-resin with trifluoroacetic acid and a cocktail of scavengers to prevent unwanted side reactions (9). Recovery of the peptide from this mixture is typically accomplished either by ether precipitation or by aqueous-ether extraction (9), both of which are somewhat labor-intensive.

An apparatus of our own design has been constructed which fully automates the side-chain deprotection and resin-cleavage of peptide-resin samples. The apparatus accommodates peptides containing trifluoroacetic acid (TFA)-labile side-chain protecting groups and resin linkages. An established cleavage protocol is used which efficiently removes all side-chain protecting groups used in Fmoc chemistry (9). A continu-

MATERIALS AND METHODS

General

High-purity dichloromethane and dimethylformamide were obtained from Burdick & Jackson and used without further purification. Trifluoroacetic acid (Fluka, Buchs, Switzerland), thioanisole (Aldrich, Milwaukee, WI), 1,2-ethanediol (Aldrich), glacial acetic acid (Baker, Phillipsburg, NJ) and phenol (Gibco BRL/Life Technologies, Gaithersburg, MD) were used without further purification.

Figure 1. The robotic peptide-resin deprotection/cleavage apparatus, consisting of two substations. The trifluoroacetic acid reactions occur in disposable polypropylene vessels in the cleavage substation (left), and the evaporation and extraction procedures occur in a glass sidearm tube in the evaporation/extraction substation (right).
Fmoc amino acids and polystyrene resins were obtained from Advanced Chemtech (Louisville, KY). Side-chain protecting groups used with the apparatus include: 1-butyl esters for Asp and Glu, 1-butyl ethers for Ser, Thr, and Tyr; triphenylmethyl for Cys, His, Asn, and Gln; 1-butoxycarbonyl for Lys; and 2,2,5,7,8-pentamethylychroman-6-sulfonyl for Arg. 4-(2,4-Dimethoxyphenyl)-Fmoc-aminomethyl)-phenoxy resin (100–200 mesh, 1% crosslinked with divinylbenzene) was used at a 0.40 mmol/g substitution level for the synthesis of C-terminal amides (11).

HPLC analysis of peptides was performed on a Rainin HPX system controller with a C18 reversed-phase HPLC column (Vydac, 25 cm x 4.6 mm; The Separations Group, Hesperia, CA) and a gradient elution (solvent A: H2O/0.1% TFA and solvent B: CH3CN/0.1% TFA; 0%–50% B in 4 min).

Amino acid compositions were obtained using the Pico-Tag method of Waters Chromatography (Div. of Millipore, Milford, MA) (1). Mass spectra were analyzed in a glycerol matrix by Liquid Matrix Secondary Ion Mass Spectrometry on a VG Analytical ZAB 2SE mass spectrometer at Mass Search (Modesto, CA).

Robotics

The cleavage/deprotection workstation was designed around a Zymate XP robot (Zymark, Hopkinton, MA), which consists of a central arm that provides a variety of functions via a gripping hand and a modified 30-ml syringe hand (Figure 2). A Remote Computer Interfaces Module Card (Zymark) connects the Zymark controller to a Macintosh II (Apple Computer, Cupertino, CA). The Macintosh is programmed in THINK C language and sends commands through its serial port to the Zymark, which then executes the robotic movements (15). The computer is equipped with a MacADIOS II multifunction analog and digital I/O board and three daughter boards (GW Instruments, Somerville, MA) which allow for the control of over 48 inputs and outputs. The Macintosh controls the solenoid valves, a pneumatic air cylinder and a diaphragm pump via the multifunction I/O board. The multifunction I/O board is connected to a circuit board containing solid-state relays (5–60 V DC; Newark, Chicago, IL) by a 34-pin ribbon cable. The MacADIOS II board triggers the relays by outputting 0 V and +5 V signals which connect the solenoid valves to a

Figure 3. The cleavage station consists of a polypropylene receptacle that forms a leak-tight seal with a disposable cleavage vessel delivered by the robot arm. Reagent mixing and draining are controlled by a solenoid valve that can open the vessel to a nitrogen source or to a drain path (C = common, NC = normally closed). A multiple-reagent spigot is used to deliver a variety of reagents and can form a gas-tight seal when pressed against the top of the cleavage vessel. Contents of the cleavage vessel can be directed to either an extraction tube or to a waste receptacle. M = Multi-port solenoid valve.

Figure 4. The evaporation/extraction station has two positions that can be vertically accessed by reagent-delivery tubes. Evaporation occurs in the heating block position (left) with the delivery of nitrogen gas. Aqueous acetic acid can also be delivered to this station. Aqueous-ether continuous extraction occurs in the adjacent position. P = Diaphragm pump.
power supply. All solenoid valves (General Valve, Fairfield, NJ) were made from Teflon® and operate at +24 V DC.

Cleavage Workstation

The cleavage workstation (Figure 3) consists of a polypropylene platform with a tapered receptacle (0.166" i.d.) that forms a leak-proof seal with luer-tipped polypropylene vessels. Disposable 15-ml polypropylene vessels are fitted with a 20-μm polyethylene frit (Varian Sample Preparation Products, Harbor City, CA) and stored in three 4" × 1" glass dispensers which vertically stack up to 16 reaction vessels each. A 2-solenoid, 3-port (normally closed) valve is located beneath the cleavage platform and can either open to nitrogen pressure to allow reagent mixing or open to an outlet to effect draining. Teflon tubing (1/16" o.d.; Cole-Parmer, Chicago, IL) directs fluid drained from the cleavage vessel to either a waste position or an evaporation/extraction tube in the collection rack. The fluid destination is controlled by the activation of a pneumatic air cylinder, resulting in movement of the collection rack (Figure 3).

Dichloromethane and acetic acid are stored in 200-ml pressurized bottles (Rainin Instrument, Woburn, MA) fitted with Teflon spigot lines. The cleavage reagents, 94% TFA/H2O and the scavenger mixture, are stored separately in 500-ml and 200-ml pressur-

ized amber bottles, respectively, fitted with Teflon spigot lines. Delivery of all four of these reagents is controlled by 2-way solenoid valves. These reagents are delivered to the cleavage vessel by pressurized Teflon lines (1/16" o.d.) which culminate in a custom-made multi-channeled spigot (Figure 3). This multi-spiugot consists of a polyethylene rod (1.0" o.d.) fitted with an internal spring and a 1/8" layer of polyethylene foam (McMaster-Carr, Los Angeles, CA) on the contacting surface. This design allows an air-tight seal to be made

![Figure 5](image.png)

**Figure 5.** Scavengers and reaction by-products are removed from an acidic aqueous peptide solution by a continuous liquid-liquid extraction procedure. Ether is delivered through a fritted glass tube, flows through the aqueous layer and carries the contaminants away via the sidearm tube.

![Figure 6](image.png)

**Figure 6.** Reversed-phase C18 analytical HPLC analysis of a series of ten crude decapeptides, deprotected/cleaved automatically by the robotic apparatus. All peptides are acetylated and amidated:

(a) AAFHTTGRII, (b) RAFHTTGRII, (c) RAHTTGRII, (d) RAPFTTGRII, (e) RAFHTAGRII, (f) RAFHTGRII, (g) RAFHTTARIII, (h) RAFHTTARII, (i) RAFHITGRII, (j) RAFHTTGRII.
with the cleavage vessel to facilitate liquid draining and compensates for any differences in vertical positions. The flow of nitrogen gas through the multi-spigot is controlled by a 2-way solenoid valve and a needle valve (Furon, Anaheim, CA).

**Evaporation/Extraction Station**

The evaporation station (Figure 4) consists of an aluminum heating block (custom made) wrapped with nichrome heating wire (Ace Glass, Vineland, NJ). A Pyrex® gas-delivery tube (25 cm x 5 mm i.d.) is located directly above the heating unit and is mounted on a movable rack controlled by a pneumatic air cylinder with a 9-inch stroke (Parker Fluidpower, Des Plaines, IL). The rack can be lowered to submerge the gas-delivery tube into a liquid sample in the evaporation/extraction tube, or raised, to allow removal of the sample tube from the station. The gas-delivery tube is connected to a nitrogen source via a male pipe adapter (1/8" o.d.) and Teflon tubing. Nitrogen gas is delivered through the glass tube by activation of a 2-way solenoid valve, and the flow rate is controlled by a needle valve. Aqueous acetic acid (10%) can also be delivered through the gas-delivery tube and is stored in a 1-liter pressurized bottle.

The extraction station consists of a solid polypropylene block that holds one evaporation/extraction tube (Figure 4). This tube is a 50-ml conical Pyrex centrifuge tube (Ace Glass) modified at the 20-ml level with a downward-sloping sidearm tube (8 mm o.d.). A funnel is located below the outlet of the sidearm and directs liquid to a waste receptacle. A Pyrex gas-dispersion tube (25 cm x 6 mm, Ace Glass) with a fritted end (20 μm) or a tapered end is located directly above the polypropylene block on the same movable rack as the gas-delivery tube. Ether is delivered through the fritted tube by a diaphragm pump (ProMinent Fluid Controls, Pittsburgh, PA) operated under computer control.

**Cleavage Protocol**

The cleavage protocol begins with the delivery of a disposable vessel from the dispenser rack to the cleavage platform by the Zymark gripping hand (Figure 2). The peptide-resin samples (ca. 50–500 mg) are transferred from a peptide synthesis station (14), or a suitable storage rack, to the cleavage station with the 30-ml syringe hand. The resin is transferred as a slow-settling slurry in 60% 1,2-dichloroethane/dimethylformamide (14). The transfer process is repeated three times to ensure quantitative transfer of the resin. The transfer solvent is then directed to waste by the application of positive nitrogen pressure by the multi-spigot (Figure 3). The resin is then rinsed with CH₂Cl₂ (1x 10 ml), dried with nitrogen (1 min), and the cleavage reagents are then delivered. The scavenger cocktail (1.0 ml, ethanedithiol:thioanisole: phenol, 1:2:2) is initially delivered, followed by the 94% TFA/H₂O solution (9 ml). The reaction is allowed to incubate for 2 h with periodic bubbling of argon through the bottom of the reaction vessel. The peptide solution is then filtered into an evaporation/extraction tube by the application of positive nitrogen pressure from the multi-spigot. The resin is washed with TFA (1x 2 ml), followed by glacial HOAc (1x 0.5 ml). The spent cleavage vessel is then removed by the gripping hand and placed into a waste receptacle.

The peptide solution is then transferred from the cleavage station to the heating unit of the evaporation workstation by the gripping hand. The TFA solution is evaporated to a volume of approximately 1 ml with a stream of nitrogen and gentle heating (ca. 40°C) for 45 min. The concentrated solution is then diluted with 10 ml of 10% aqueous acetic acid and mixed thoroughly by nitrogen bubbling to form a uniform suspension. The extraction/evaporation tube is then transferred to the extraction station with the gripping hand.

Removal of the scavengers and protecting group by-products is performed by continuous aqueous-ether extraction (Figure 5). The fritted (or tapered) glass tube is positioned at the bottom of the peptide suspension, and diethyl ether or methyl tert-butyl ether is delivered for 45 min at a flow rate of approximately 2 ml/min. During the continuous-extraction process, excess ether overflows through a sidearm tube and is directed to waste. The extraction tube is then removed by the gripping hand and placed into a storage rack, where the ether is allowed to evaporate. The peptide solution is then ready to be frozen and lyophilized.

The evaporation and extraction workstations are rinsed between samples to avoid cross-contamination. A clean rinse tube is placed into the evaporation station, and 10% HOAc/H₂O is delivered through the gas-delivery tube. The gas-delivery tube is rinsed thoroughly by bubbling this solution (2x 2 min). This procedure is repeated with a fresh aliquot of solution. The rinse tube is then transferred to the extraction station, where ether is delivered for 20 min to rinse the fritted ether-dispersion tube.

### Table 1. Yields of Automatically Deprotected/Cleaved Peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Weight (mg)</th>
<th>Yield (%)</th>
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<tbody>
<tr>
<td>AFHTGRII</td>
<td>30</td>
<td>49</td>
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<tr>
<td>RAFHTGRII</td>
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<tr>
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<tr>
<td>RAFFTAGRII</td>
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<td>49</td>
</tr>
<tr>
<td>RAFFTGRAI</td>
<td>47</td>
<td>77</td>
</tr>
</tbody>
</table>

**Figure 7.** Reversed-phase C18 HPLC analysis of the scavenger cocktail under the same gradient conditions as the peptide chromatograms shown in Figure 6, indicating the effective removal of scavengers by the robotic cleavage/deprotection procedure.

**RESULTS**

The cleavage/deprotection apparatus was tested by determining the yield and purity of a set of ten decapeptides. These peptides were synthesized by Fmoc chemistry (3,14) on a TFA-labile Rink amide resin (11). The average peptide yield after the cleavage/
Table 2. Characterization of Automatically Deprotected/Cleaved Peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>[M+H]^+ m/z</th>
<th>Amino Acid Analysis</th>
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<tr>
<td></td>
<td>calc.d.</td>
<td>obs.</td>
</tr>
<tr>
<td>AAFHTGRII</td>
<td>1127.5</td>
<td>1127.6</td>
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<td>RAFHTGRII</td>
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<td>1146.6</td>
</tr>
<tr>
<td>RAFHATGRII</td>
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<td>1182.6</td>
</tr>
<tr>
<td>RAHTTGRAI</td>
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<tr>
<td>AAFHTTGRAI</td>
<td>1226.7</td>
<td>1226.3</td>
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<tr>
<td>RAFFTGR1A</td>
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<td>RAFFTGR1A</td>
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<tr>
<td>RAFFTGR1A</td>
<td>1170.7</td>
<td>1170.7</td>
</tr>
</tbody>
</table>

deprotection of 100-mg peptide-resin samples was 65% of theoretical (Table 1). The purity of the peptides was >90% as determined by reversed-phase HPLC (Figure 6). The identity of each major peak (by HPLC) was confirmed by amino acid analysis and mass spectrometry (Table 2). These chromatograms indicate that the automated method quantitatively removes the scavengers; an analysis of the scavenger cocktail under identical HPLC conditions is shown for comparison (Figure 7).

**DISCUSSION**

A robotic workstation for the deprotection and cleavage of peptides with TFA-labile side chains and resin linkers has been designed and constructed around a Zymark robot. This robotic system has been previously adapted to multiple-peptide and equimolar-peptide mixture (EPM) synthesis (14). The workstation described here interfaces with the EPM synthesizer, allowing for postsynthesis peptide cleavage and deprotection without user intervention.

Special features of the apparatus have been designed which allow the deprotection/cleavage protocol to be efficiently automated:

1. The TFA deprotection/cleavage reactions are performed in fritted, disposable vessels. This allows the acid treatment and subsequent filtration step to be performed in the same vessel, and eliminates cross-contamination.

2. A receptacle for the disposable vessels was designed to easily form a leak-proof seal with the vessel upon delivery by the robot arm. The ability of the robot to sense vertical pressure is used to establish a successful fit.

3. Reagent mixing in the cleavage vessel can then be achieved by energizing one port of a solenoid valve. This opens the cleavage vessel to nitrogen pressure and thoroughly agitates the resin slurry periodically during the 2-h incubation period. Evaporation of TFA during this period is kept to a minimum by mixing for only 5 out of every 30 seconds.

4. Liquid draining occurs by energizing the other port of the solenoid valve, which directs the fluid to either a waste or collection vessel. The multi-spiogt ensures that reagents are drained quickly and reproducibly by forming a seal with the top of the cleavage vessel.

5. In addition to its pressure-sealing capability, the multi-spiogt configuration allows for the rapid delivery of several reagents used at the cleavage workstation, thereby minimizing time and the number of robotic movements required. For example, the cleavage/deprotection reagents, which are stored as two separate solutions in order to increase the reagent's stability, are added in rapid succession with the multi-spiogt.

6. The TFA-evaporation and ether-extraction steps are performed in the same tube, minimizing losses due to sample transferring. The evaporation time (45 min) is controlled such that the residue does not reach dryness, in order to facilitate peptide dissolution.

7. The overall processing time is minimized by performing an evaporation and extraction step simultaneously with a cleavage reaction. Since the rate-limiting step is the 2-h TFA cleavage/deprotection reaction, the preceding sample is taken through the evaporation step (45 min) and the extraction step (45 min) during this time. The remaining 30 min are used to rinse the evaporation/extraction station. In this manner, a sample is processed every 2 h. The flexibility of the software can accommodate longer cleavage times as needed.
The productivity of the apparatus could be substantially increased by expanding the workstation’s capacity to allow the processing of several samples in parallel. The flexibility of this robotic system can also allow the inclusion of on-line HPLC analysis.

In conclusion, an apparatus has been constructed which automates the cleavage and deprotection of peptides synthesized by FMOC chemistry. In addition to the automation of an otherwise laborious procedure, the described apparatus minimizes human contact with the noxious cleavage reagents. The workstation processes between 50–500 mg of resin sample every 2 h and provides the peptides in 50%–80% yield as an aqueous acidic solution. More consistent yields are likely to be obtained (especially with hydrophobic peptide sequences) when cleavage/deprotection strategies are used that do not involve extraction or precipitation, such as the incorporation of photolabile (7) or diketopiperazine-forming (2) resin linkers, or the use of affinity-based protecting groups (4,10). This instrument has greatly increased the rate of peptide cleavage/deprotection in the processing of hundreds of peptides in our laboratories to date.

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REFERENCES


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