

# Preparation of the caspase-3/7 substrate Ac-DEVD-pNA by solution-phase peptide synthesis

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**This protocol describes the gram-scale solution-phase synthesis of the colorimetric caspase-3/7 substrate Ac-DEVD-pNA. The caspase enzymes are integral to cellular inflammation and apoptotic cascades, and are commonly studied by cell biologists, medicinal chemists and chemical biologists. In particular, the assessment of caspase enzymatic activity is a standard method to evaluate cell death pathways and new apoptosis-modulating agents. Caspase enzymatic activity can be conveniently monitored with peptidic chromogenic or fluorogenic substrates, with certain peptide sequences imparting selectivity for certain caspases. The synthesis of these peptide substrates is typically carried out by solid-phase synthesis, a method that is not ideal for production of the gram quantities needed for high-throughput screening. Described herein is a facile method for the synthesis of the Ac-DEVD-pNA caspase-3/7 substrate using solution-phase peptide synthesis. This protocol, involving iterative PyBOP-mediated couplings and Fmoc deprotections, is rapid (about 5 d), operationally simple and can be used to generate over 1 g of product at a fraction of the cost of the commercial substrate.**

## INTRODUCTION

Caspases are a group of cysteine proteases that have critical roles in the inflammatory response (caspases-1, -4 and -5) and apoptotic cascade (caspases-2, -3, -6, -7, -8, -9, -10 and -12)<sup>1</sup>. Caspases reside in the cell as low-activity zymogens called procaspases<sup>2</sup>. During apoptosis, a caspase activation cascade begins with a signal that activates an ‘initiator’ procaspase to the active caspase enzyme, with procaspase-8 and -10 being activated through the dimerization of cellular death receptors<sup>3</sup> and procaspase-9 being activated through cytochrome c release from the mitochondria and formation of the apoptosome<sup>4,5</sup>. Once activated, caspase-8 and caspase-9 catalyze the hydrolysis of procaspase-3 and procaspase-7, forming the ‘executioner’ caspases-3 and -7 (refs. 6–8). Caspase-3 (and to a lesser extent caspase-7) is responsible for the proteolytic cleavage of over 100 different protein substrates<sup>9,10</sup>, resulting in the systematic dismantling of the cell and apoptosis (Fig. 1).

Given their central role in apoptotic cell death, aberrant caspase activity can have disastrous consequences for an organism. For example, in many degenerative diseases, an increase in caspase activity is observed and is believed to lead to premature cell death. Evidence for elevated caspase activity has been seen in Alzheimer’s disease<sup>11–13</sup>, Parkinson’s disease<sup>12,14</sup>, Huntington’s disease<sup>15</sup>, ALS<sup>16</sup> and several others. In these cases, caspase inhibitors have been long sought as potential cytoprotective agents and indeed the first caspase inhibitors are now in clinical trials for the treatment of liver disease, myocardial infarction and rheumatoid arthritis<sup>17</sup>. Conversely, resistance to apoptosis is a hallmark of cancer<sup>18</sup> and cancer cells can evade apoptosis through a modulation in caspase activity. Caspase-8 (refs. 19–24) and -10 (refs. 25,26) are mutated in some cancers, and the elevated levels of endogenous caspase-inhibitor proteins (e.g., XIAP and survivin) are commonly observed in a variety of cancers<sup>27–29</sup>. It is interesting to note that the levels of procaspase proteins are elevated in many tumor types<sup>30–35</sup> and this has led to the hypothesis that drugs that directly activate procaspase enzymes could selectively induce apoptotic death in cancer cells<sup>35–37</sup>.

Shortly after the discovery of the caspases, a series of elegant studies defined the preferred cleavage site for each caspase isozyme<sup>38,39</sup>.

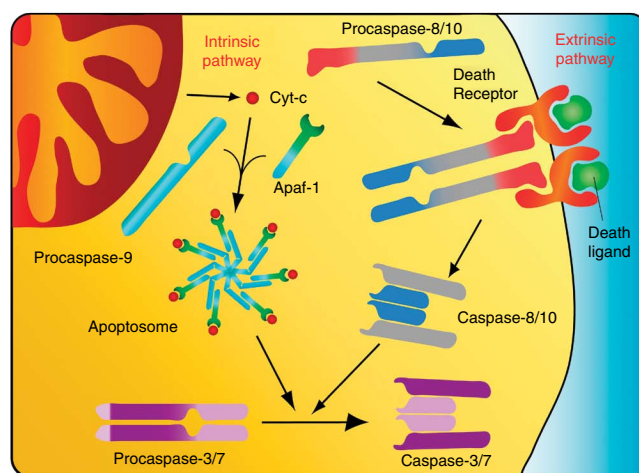
As indicated by their name (*cysteine aspartic acid protease*), caspases recognize short peptidic sequences and universally cleave on the C-terminal side of aspartic acid residues. The substrate recognition sequence for each caspase has been largely elucidated; for the most part, these enzymes recognize tetrapeptide sequences. The discovery of the preferred recognition sequence for each caspase led directly to the development of peptidic inhibitors and substrates for each isozyme. In the development of inhibitors<sup>39,40</sup>, an electrophilic ‘warhead’ (typically an aldehyde or  $\alpha$ -fluoro ketone) is appended to the carboxy terminus of the scissile aspartic acid. Likewise, substrates can be created by appending the appropriate chromophore or fluorophore to this same position. The use of the *p*-nitroaniline (pNA) chromophore is especially popular and as such the pNA tetrapeptide substrates for caspases-1, -3, -6, -8 and -9 are available from many commercial vendors.

Caspase-3, with its multiple cellular substrates, is one of the most studied and important caspases. Procaspase-3 is activated to caspase-3 through the action of either caspase-8 or caspase-9, meaning that procaspase-3 is a lynchpin upon which both the death receptor and mitochondrial apoptotic pathways converge (Fig. 1)<sup>3</sup>. As such, there is much interest in and usage of the preferred peptidic substrates for caspase-3, Ac-DEVD-pNA (colorimetric) and Ac-DEVD-AMC (fluorogenic; AMC is 7-amino-4-methylcoumarin) in cell biology, chemical biology and medicinal chemistry. For example, Ac-DEVD-pNA and Ac-DEVD-AMC are widely used to monitor caspase-3/7 activity in cell culture, have been used for the *in vitro* identification of caspase-3/7 inhibitors<sup>40–51</sup>, and in the identification and assessment of small molecule activators of procaspase-3/7 (refs. 35–37). Caspase-3 or caspase-7 will catalyze the hydrolysis of Ac-DEVD-pNA, releasing the pNA chromophore which has a  $\lambda_{\text{max}}$  of 405 nm (Fig. 2).

Although the average cost of Ac-DEVD-pNA from commercial vendors (5 mg for ~\$60) is reasonable when small quantities of this reagent are needed, it is prohibitively expensive to purchase the gram quantities required to screen a small molecule library for caspase inhibition/activation. For example, to screen a 200,000



**Figure 1** | Pathways of caspase activation. Release of pro-apoptotic proteins (such as cytochrome *c*) from the mitochondria promotes the formation of the apoptosome, a multimeric complex including Apaf-1, cytochrome *c* and procaspase-9. In the apoptosome, procaspase-9 becomes activated to caspase-9, an enzyme that can then proteolytically cleave procaspases-3 and -7, resulting in their activation. In an analogous manner, procaspases-8 and -10 are activated to caspases-8 and -10 after death ligands bind to surface death receptors, initiating the dimerization and recruitment of procaspase-8 and -10. Once activated, caspases-8 and -10 can also proteolytically cleave procaspases-3 and -7 to form the active caspases-3 and -7.



compound library for caspase-3 inhibition under standard 384-well plate assay conditions would require ~1.25 g of Ac-DEVD-pNA. Described herein is a protocol to synthesize 1 g of this Ac-DEVD-pNA substrate. Through this method, Ac-DEVD-pNA can be produced for approximately one-fiftieth of the commercial cost using rapid, continuous solution-phase peptide synthesis<sup>52,53</sup>. Ac-DEVD-pNA synthesized through this method has been used to generate substrate for the assessment of caspase inhibitors<sup>40</sup>, for the high-throughput screening for procaspase-3 activators<sup>35</sup>, and for mechanistic studies on procaspase-3-activating compounds<sup>36,37</sup> and cytoprotective agents<sup>54</sup>.

### Comparison between solution-phase and solid-phase synthesis

Standard solid-phase peptide syntheses involve coupling to a resin, followed by C- to N-terminal peptide construction in an iterative sequence involving deprotection, purification and coupling. Purification of products is achieved by filtration, where impurities are washed through a filter, which retains the solid support and the growing peptide chain. The ability to filter the product is advantageous when synthesizing longer peptides and facilitates automation of solid-phase peptide synthesis. As the peptide is formed on a solid support and not in solution, coupling reactions are not as efficient and require large excess of each amino acid (as high as 5–40 equivalents). Furthermore, owing to low loading efficiency on the solid support, a large amount of resin is often required to produce gram quantities of the final peptide. The solution-phase synthesis described herein is advantageous when large quantities of a relatively short peptide are desired. In this example, gram quantities of the Ac-DEVD-pNA caspase-3/7 substrate can be synthesized, in good overall yield, using nearly stoichiometric quantities of reagents (the amino acids are used in only slight excess, 1.1 equivalents). This route also allows for the addition of the chromophore before couplings, thereby enabling the purification of this product to be carried out early in the synthesis. In contrast, in solid-phase synthesis the protected peptide is typically cleaved from the resin before being coupled to the chromophore.

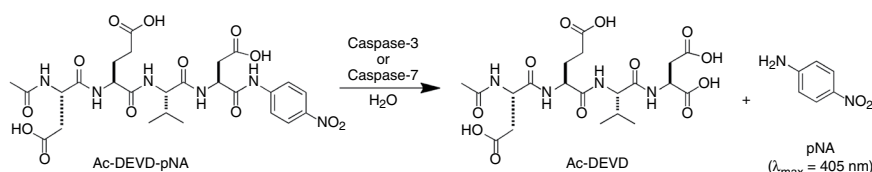
### Experimental design

As shown in **Figure 3**, the Ac-DEVD-pNA peptide is synthesized from C- to N-terminus. The synthetic route begins by coupling pNA to

Fmoc-Asp(O*t*-Bu)-OH using POCl<sub>3</sub> (ref. 55). After workup, the resulting Fmoc-Asp(O*t*-Bu)-pNA amide (**1** in **Fig. 3**) is purified by silica gel and isolated in 77% yield. This product is then carried through iterative Fmoc deprotection, amino acid coupling and workup steps without any intermediate purification; protected valine is first coupled to provide **2**, then Fmoc deprotection and PyBOP coupling with protected glutamic acid provides **3**, and finally Fmoc deprotection and coupling with acylated aspartic acid provides the fully protected peptide **4**. This protected peptide is difficult to isolate by extraction and column chromatography. Attempted extraction of the resulting product into ethyl acetate (EtOAc), chloroform, methylene chloride or hexane results in a persistent emulsion and an insoluble precipitate. Efforts to purify the crude material using column chromatography result in an insoluble material that precipitates on the column. However, this peptide can be isolated by trituration in diethyl ether. Protected peptide **4** is then globally deprotected using trifluoroacetic acid (TFA) to provide the final product. The product is isolated by trituration in diethyl ether to give an overall yield of 40% for the three couplings and the global deprotection.

This procedure has been performed on a 1–10 mM scale with minimal differences in protocol. When working on smaller scales, reaction times may be decreased to 20 min per coupling/deprotection step. Conversely, larger scales may require longer reaction times and should be monitored to verify completion. Although the synthesis reported herein is specific for the Ac-DEVD-pNA substrate, analogous strategies could likely be used to produce other caspase substrates, inhibitors or reporters including, Ac-DEVD-AFC, Ac-DEVD-AMC, FAM-DEVD-fmk and others. Individuals wishing to carry out such modified protocols should ensure that there is a free amine on the chromophore or fluorophore of choice and the initial coupling reaction to the protected aspartic acid residue may require optimization. Owing to the relatively low nucleophilicity of pNA, the strong activator POCl<sub>3</sub> was used in the Ac-DEVD-pNA protocol described herein. For other reporters, different coupling methods may be more suitable. After coupling to aspartic acid, an analogous protocol would be followed for the remaining coupling/deprotection steps.

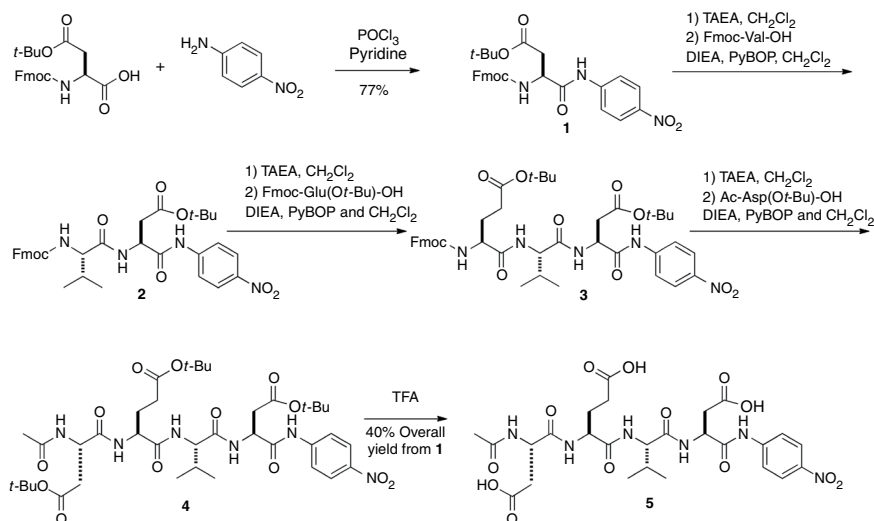
The method outlined in **Figure 3** represents an improvement over our previously reported synthesis<sup>40</sup>, as the earlier protocol



**Figure 2** | The hydrolysis of Ac-DEVD-pNA to Ac-DEVD and *p*-nitroaniline (pNA), as catalyzed by caspase-3 or caspase-7.

## PROTOCOL

requires an extraction (after final coupling) that results in an emulsion that is difficult to resolve. The method reported herein takes advantage of the insolubility of the product in common solvents in the utilization of precipitation-based purifications. We anticipate this method and those related to it will be useful for the preparation of large quantities of caspase substrates required for high-throughput screening.



**Figure 3** | Solution-phase synthesis of Ac-DEVD-pNA.

## MATERIALS

### REAGENTS

- Brine solution, saturated NaCl solution (Fisher Scientific, cat. no. S271)
- Sodium bicarbonate, saturated solution (Fisher Scientific, cat. no. S233)
- Sodium sulfate, anhydrous (Sigma-Aldrich, cat. no. 239313)
- Acetone (Sigma-Aldrich, cat. no. 179124)
- Methylene chloride (Sigma-Aldrich, cat. no. D65100) **! CAUTION** Irritant, wear gloves and avoid long-term exposure.
- Methanol (Fisher Scientific, cat. no. A412) **! CAUTION** Poisonous, highly flammable, avoid long-term exposure.
- Hexanes (Fisher Scientific, cat. no. H303) **! CAUTION** Highly flammable, avoid long-term exposure and environmental toxin.
- EtOAc (Sigma-Aldrich, cat. no. 319902)
- Diethyl ether (Fisher Scientific, cat. no. E138)
- *N,N*-diisopropylethylamine, DIEA, Hünig's base (Sigma-Aldrich, cat. no. D12, 580-6) **! CAUTION** Highly flammable, corrosive and avoid exposure.
- Tris(2-aminoethyl)amine (TAEA; Sigma-Aldrich, cat. no. 225630) **! CAUTION** Corrosive, hygroscopic, handle with care.
- TFA (Sigma-Aldrich) **! CAUTION** Corrosive, handle with care.
- Phosphate buffer
- pNA (Aldrich) **! CAUTION** pNA is toxic. Use proper protective equipment when handling this compound.
- Ac-Asp(Ot-Bu)-OH (ChemImpex, cat. no. 04343)
- Fmoc-Asp(Ot-Bu)-OH (Novabiochem, cat. no. 04-12-1013)
- Fmoc-Glu(Ot-Bu)-OH (Novabiochem, cat. no. 04-12-1020)
- Fmoc-Val-OH (Novabiochem, cat. no. 04-12-1039)
- PyBOP (Novabiochem, cat. no. 01-62-0016) **! CAUTION** Irritant, avoid contact.
- Pyridine (EMD, cat. no. PX2020-6) **! CAUTION** Pyridine is toxic. Avoid breathing the fumes. Work with pyridine in a chemical fume hood and cover reaction vessels with septum when moving between the hood and the rotary evaporator to minimize the spread of vapors.
- Ninhydrin TLC stain
- DPIP TLC stain
- NaCl (Fisher Scientific, cat. no. S271)
- POCl<sub>3</sub> (Sigma-Aldrich, cat. no. 201170) **! CAUTION** POCl<sub>3</sub> is corrosive. Avoid contact with skin.

### EQUIPMENT

- Büchner funnel (CoorsTek, cat. no. 60243)
- One-necked 500 ml round-bottom flasks (Chemglass)
- 500 ml Erlenmeyer flasks (Pyrex)

## PROCEDURE

### Recrystallization of pNA

**1** | Add 10 g of pNA to a 500 ml Erlenmeyer flask. Add 450 ml of H<sub>2</sub>O. Boil on a stirring hot plate with a stir bar to completely dissolve all the solid material. If needed, add additional H<sub>2</sub>O until all solid has been dissolved.

**! CAUTION** pNA is toxic. Use proper protective equipment when handling this compound.

**2** | Set up a hot filter by placing a funnel over an Erlenmeyer flask with ~10 ml of boiling H<sub>2</sub>O. Place a fluted piece of filter paper into the funnel and wet with a small volume of H<sub>2</sub>O (~5 ml). Allow the funnel and filter paper to heat in the steam from the Erlenmeyer flask.

- 250 ml Erlenmeyer flask (Pyrex)
- Filter paper (Whatman)
- Glass funnel (Pyrex)
- 150 ml fritted filter funnel with coarse frit (Chemglass)
- Glass stoppers (Pyrex)
- 50 ml Graduated cylinder (Kimax)
- High vacuum pump (Welch)
- Laboratory clamps (Fisher Scientific)
- Laboratory jacks (Fisher Scientific)
- Magnetic stir/hot plate (Corning)
- Rotary evaporator (Büchi)
- 1,000 ml Separatory funnel (Pyrex or Kimax)
- 500 ml Separatory funnel (Pyrex or Kimax)
- Small stir bars (Fisher Scientific)
- 4-Vane stir bar (Fisher Scientific)
- Rubber septa (Chemglass)
- 100 cm × 190 cm recrystallization dish (Kimax)
- Thermometer (–20 to 20 °C)
- 50 ml disposable syringe (Henke Sass Wolf)
- 1 ml disposable syringe (Henke Sass Wolf)
- 5 ml disposable syringe (Henke Sass Wolf)
- 100 ml beaker (Pyrex)
- Silica gel (Silicycle)
- TLC plates (Silicycle)

### REAGENT SETUP

**Phosphate buffer** Phosphate buffer is 90 g NaHPO<sub>4</sub>·H<sub>2</sub>O (EMD 567549) and 37.5 g NaH<sub>2</sub>PO<sub>4</sub> (EMD 567547) (Sigma-Aldrich) in 500 ml H<sub>2</sub>O pH 5.5.

**Hünig's base** Hünig's base was dried by distillation over CaH<sub>2</sub> immediately before use. An appropriate volume of Hünig's base was placed into a distillation flask, CaH<sub>2</sub> was added, the flask was heated and the distillate was collected. Alternatively, a sure-seal bottle of Hünig's base may be purchased and used.

### EQUIPMENT SETUP

**Glassware** Thoroughly clean and dry overnight (12 h) all glasswares in an oven (100 °C) before use.

- 3| Filter the hot pNA solution through the hot filter to remove any insoluble particulates.
  - 4| Boil the filtrate until crystals begin to form. Immediately remove from heat and allow it to cool completely in an undisturbed location. Long yellow needles will be formed.
  - 5| Filter the crystals through a piece of filter paper to obtain pure crystals. Wash the crystals with two washes (20 ml) of ice cold H<sub>2</sub>O. Allow the crystals to air dry on a filter paper.
- **PAUSE POINT** The recrystallized pNA can be stored at room temperature for at least 1 year.

#### Coupling of pNA to Fmoc-Asp(OtBu)-OH

- 6| To a clean, dry, 500 ml round-bottom flask add 4.115 g (10 mmoles) of Fmoc-Asp(Ot-Bu)OH, 1.381 g (10 mmoles) of pNA and 30 ml of pyridine. Add a stir bar.
 

! **CAUTION** Pyridine is toxic. Avoid breathing the fumes. Work with pyridine in a chemical fume hood and cover reaction vessels with septum when moving between the hood and the rotary evaporator to minimize the spread of vapors.
- 7| Make a salt-ice bath by placing a large 4-vane stir bar at the bottom of a 100 cm × 190 cm recrystallization dish. Fill the dish with ice and pour 200 g of NaCl over the ice. Place the dish on a stir plate in a chemical fume hood and stir until the bath reaches a temperature of -10 to -15 °C as monitored with a thermometer.
- 8| Place the round-bottom flask containing the Fmoc-Asp(Ot-Bu)OH, pNA and pyridine into the salt-ice bath with the neck clamped to a support in the hood and the flask submerged 1.5 inches in the ice-water-salt mixture.
- 9| Slowly add 1.02 ml (11 mmoles) of POCl<sub>3</sub> through a syringe and stir for 30 min while maintaining the salt-ice bath in the -10 to -15 °C range.
 

! **CAUTION** POCl<sub>3</sub> is corrosive. Avoid contact with skin.
- 10| Quench the reaction by adding 100 ml of ice water. Fill a 100 ml beaker with ice. Add water till the 100 ml mark. Slowly pour ice and water into the reaction vessel and allow to stir for 1 min. The quenching reaction is exothermic and results in the release of HCl.
- 11| Pour the contents of the reaction vessel into a 500 ml separatory funnel. Add 150 ml of EtOAc. Extract the reaction mixture. The product will be extracted into the organic (upper) phase.
 

! **CAUTION** Pressure can build up in the separatory funnel during extraction. Vent the separatory funnel several times during shaking by turning the separatory funnel upside down and opening the stopcock to release built up air pressure.
- 12| Drain the aqueous (lower) phase into a 250 ml Erlenmeyer flask. Pour the organic phase into a separate 500 ml Erlenmeyer flask.
- 13| Pour the aqueous phase back into the separatory funnel and extract two more times, as above, with 150 ml of EtOAc by combining the organic phases.
- 14| Pour the three collected organic phases (~450 ml) into a 1 liter separatory funnel. Add 150 ml of a saturated bicarbonate solution and shake vigorously, venting the funnel frequently. Allow the phases to separate (it may take up to 2 h for this separation) and remove the aqueous phase.
- 15| Add 150 ml of brine to the separatory funnel and shake. Allow the phases to separate and remove the aqueous phase.
- 16| Pour the organic phase into a 1 liter Erlenmeyer flask and add 20-30 g of solid anhydrous sodium sulfate to remove any residual water. The sodium sulfate should be free flowing in the bottom of the flask.
- 17| Pour the dried organic phase through a fluted filter paper in a funnel and collect the filtrate in a clean dry round-bottom flask.
- 18| Concentrate the solution to <100 ml by rotary evaporation.

## PROTOCOL

**19|** Attach a large 150 ml fritted filter funnel with a coarse frit to a clamp in a fume hood. Fill the filter 2" high with silica gel. Pour the concentrated product over the 2" silica pad and elute the product with a 3:1 mixture of hexane–EtOAc under mild vacuum. The product should elute in the first 300 ml and the impurities such as pyridine or the unreacted starting materials should stay on the column. The elution of product may be monitored by TLC.  $R_f = 0.71$ .

**20|** To remove residual pyridine, the reaction mixture should be co-evaporated in a series of solvents. Concentrate to near dryness using a rotary evaporator. Redissolve the product in 50 ml of toluene to create an azeotrope with the pyridine. Concentrate this solution to near dryness. Add 50 ml of EtOAc. Concentrate to dryness. Add 50 ml of MeOH. Concentrate to dryness. The product will be a yellow residue until the final evaporation, at which point it will be a yellow to white powder. The theoretical yield for this step should be 5.316 g of pure product.

■ **PAUSE POINT** This is a good stopping point. The purified product can be stored in solid form, at room temperature, protected from light, for up to 6 months with no observable degradation. The following steps through the workup of Ac-DEVD-pNA should be completed during the course of 1 d.

### Deprotection of Fmoc-Asp(Ot-Bu)-pNA

**21|** Dissolve 2.658 g (5 mmoles) of the Fmoc-Asp(Ot-Bu)-pNA in 30 ml of methylene chloride in a 500 ml round-bottom flask. Add a stir bar and clamp to a support in a chemical fume hood directly over a stir plate. Add 30 ml (200 mmoles) of TAEA slowly to the reaction vessel. Allow stirring for 30 min.

**22|** Monitor the reaction by TLC to verify conversion. The TLC should be run in 10% MeOH in EtOAc. The TLC can be stained with Ninhydrin to visualize the free amines that are exposed during deprotection. Dip the TLC plate into the Ninhydrin stain and heat with a heat gun until color is exposed.  $R_f=0.43$ .

### Workup

**23|** After conversion has been verified, add 60 ml of EtOAc to the reaction and then wash the reaction mixture with 50 ml of brine in a separatory funnel.

**24|** Extract the reaction mixture three times with 50 ml of phosphate buffer. Discard the aqueous fractions and dry the organic layer over anhydrous sodium sulfate using 20–30 g as in Step 16.

**25|** Concentrate to dryness with a rotary evaporator. The product should be a fluffy yellow to white powder. The theoretical yield should be 1.547 g.

### PyBOP coupling of Fmoc-Val-OH to Asp(Ot-Bu)-pNA

**26|** Redissolve the product carried over from the previous step in 30 ml of methylene chloride in a 500 ml round-bottom flask with stirring. Add 1.867 g of Fmoc-Val-OH (5.5 mmoles) to the reaction vessel.

▲ **CRITICAL STEP** Steps 27 and 28 must be done as close in time as possible to prevent epimerization. Weigh out the PyBOP for Step 28 before adding DIEA in Step 27.

**27|** Add 1.3 ml of DIEA (7.5 mmoles) to the reaction vessel slowly through syringe.

**28|** Add 2.861 g (5.5 mmoles) of PyBOP and stir reaction for 30 min. The reaction should turn from cloudy to a clear yellow over the course of a 30 min reaction.

**29|** Verify coupling by TLC. The TLC should be run in 10% MeOH in EtOAc. The TLC may be stained with Ninhydrin to verify the absence of the free amine, thus indicating stoichiometric conversion.  $R_f=0.51$ .

### Deprotection of Fmoc-Val-Asp(Ot-Bu)-pNA

**30|** After verification of coupling, add 40 equivalents of TAEA (200 mmoles, 30 ml) through syringe to the reaction mixture to deprotect the N-terminal amino acid. Allow the reaction to stir for 30 min at room temperature. The reaction should turn cloudy over the course of a 30 min reaction.

**31|** Verify deprotection by TLC. The deprotection can be detected by the presence of the free amine using the Ninhydrin stain.  $R_f=0.33$ .

**Workup**

32| After verifying that the deprotection has come to completion, add 30 ml of EtOAc.

33| Transfer the reaction to separatory funnel and wash with 50 ml of brine. Allow phases to separate.

34| Extract the reaction three times with 50 ml of phosphate buffer. Discard the aqueous phases and dry the organic phase over anhydrous sodium sulfate using 20–30 g as in Steps 16 and 24.

35| Concentrate to dryness with a rotary evaporator. The product should be a yellow residue.

**Coupling of Fmoc-Glu(Ot-Bu)-OH to Val-Asp(Ot-Bu)-pNA**

36| Add 30 ml of methylene chloride to the residue obtained above and stir to dissolve.

37| Add 2.341 g (5.5 mmoles) of Fmoc-Glu(Ot-Bu)-OH to the reaction vessel.

▲ **CRITICAL STEP** Steps 38 and 39 must be done as close in time as possible to prevent epimerization. Weigh out the PyBOP for Step 39 before adding DIEA in Step 38.

38| Add 1.3 ml of DIEA (7.5 mmoles) to the reaction vessel through syringe.

39| Add 2.862 g of PyBOP (5.5 mmoles) to the reaction vessel and stir for 30 min.

40| Verify coupling by TLC in 10% MeOH in EtOAc. Stain with Ninhydrin to verify the absence of any free amine. Rf=0.45.

**Deprotection of Fmoc-Glu(Ot-Bu)-Val-Asp(Ot-Bu)-pNA**

41| After verification of coupling, add 40 equivalents of TAEA (200 mmoles, 30 ml) through syringe to the reaction mixture to deprotect the N-terminal amino acid. Allow the reaction to stir for 30 min at room temperature.

42| Verify deprotection by TLC. The deprotection can be detected by the presence of the free amine using the Ninhydrin stain. Rf=0.42.

**Workup**

43| After verifying the deprotection has come to completion, add 30 ml of EtOAc.

44| Transfer the reaction to separatory funnel and wash with 50 ml of brine.

45| Extract the reaction three times with 50 ml of phosphate buffer. Discard the aqueous phases and dry the organic phase over anhydrous sodium sulfate using 20–30 g as in Steps 16, 24 and 34.

46| Concentrate to dryness with a rotary evaporator. The product should be yellow residue.

**Coupling of Ac-Asp(Ot-Bu)-OH to Glu(Ot-Bu)-Val-Asp(Ot-Bu)-pNA**

47| Add 30 ml of methylene chloride to the residue obtained above and stir to dissolve.

48| Add 1.272 g (5.5 mmoles) of Ac-Asp(Ot-Bu)-OH to the reaction vessel.

▲ **CRITICAL STEP** Steps 49 and 50 must be done as close in time as possible to prevent epimerization. Weigh out the PyBOP for Step 50 before adding DIEA in Step 49.

49| Add 1.3 ml of DIEA (7.5 mmoles) to the reaction vessel through syringe.

50| Add 2.862 g (5.5 mmoles) of PyBOP to the reaction vessel. Allow stirring at room temperature (22–25 °C) for 30 min. Take a TLC to verify coupling in 10% methanol in EtOAc. Stain the TLC plate with DPIP. Rf=0.65.

**Workup of protected Ac-DEVD-pNA**

51| Concentrate the reaction mixture to dryness using a rotary evaporator.

52| Add 60 ml of diethyl ether and triturate the solid product, washing the solid by swirling the flask. If necessary, pipette the ether over the solid several times to ensure good mixing.

## PROTOCOL

**53|** Filter and collect the precipitate. The impurities such as HOBT and the phosphoamide byproducts of the PYBOP coupling will be soluble in the diethyl ether. Wash the precipitate with 15 ml of diethyl ether. Allow the precipitate to dry and transfer to a round-bottom flask.

**54|** Allow to dry under vacuum overnight. The product should be a white/off-white powder.

■ **PAUSE POINT** At this point, the solid may be stored at room temperature for a week or at  $-20\text{ }^{\circ}\text{C}$  for at least 6 months.

### Global deprotection

**55|** Add 30 ml of TFA through syringe to the solid product and stir for 30 min to globally deprotect the fully protected product.

**56|** Remove the TFA by rotary evaporation.

**57|** Triturate the solid with 30 ml of diethyl ether, washing the solid by swirling the flask. If necessary, pipette the ether over the solid several times to ensure good mixing. Allow the precipitate to settle to the bottom and decant the diethyl ether. Repeat two times.

**58|** Allow the final product to dry under vacuum overnight. The product is a light tan solid.

**59|** Assess the product by  $^1\text{H}$  NMR, mass spectrometry and/or HPLC to verify proper synthesis and complete deprotection. The product appears as a single peak on an analytical reverse-phase HPLC (see **Fig. 4a**). The product should be stored at  $-20\text{ }^{\circ}\text{C}$  and is stable for at least 1 year.

### ● TIMING

Steps 1–5, Recrystallization of pNA: 4 h

Steps 6–20, Coupling pNA to Fmoc-Asp(Ot-Bu)-OH: 6 h

Steps 21 and 22, Deprotection of Fmoc-Asp(Ot-Bu)-pNA: 0.5 h

Steps 23–25, Workup of Asp(Ot-Bu)-pNA: 1 h

Steps 26–29, PyBOP coupling of Fmoc-Val-OH to Asp(Ot-Bu)-pNA: 0.5 h

Steps 30 and 31, Deprotection of Fmoc-Val-Asp(Ot-Bu)-pNA: 0.5 h

Steps 32–35, Workup of Val-Asp(Ot-Bu)-pNA: 1 h

Steps 36–40, Coupling of Fmoc-Glu(Ot-Bu)-OH to Val-Asp(Ot-Bu)-pNA: 0.5 h

Steps 41 and 42, Deprotection of Fmoc-Glu(Ot-Bu)-Val-Asp(Ot-Bu)-pNA: 0.5 h

Steps 43–46, Workup of Glu(Ot-Bu)-Val-Asp(Ot-Bu)-pNA: 1 h

Steps 47–50, Coupling of Ac-Asp(Ot-Bu)-OH to Glu(Ot-Bu)-Val-Asp(Ot-Bu)-pNA: 0.5 h

Steps 51–54, Workup of protected Ac-DEVD-pNA: 15 h

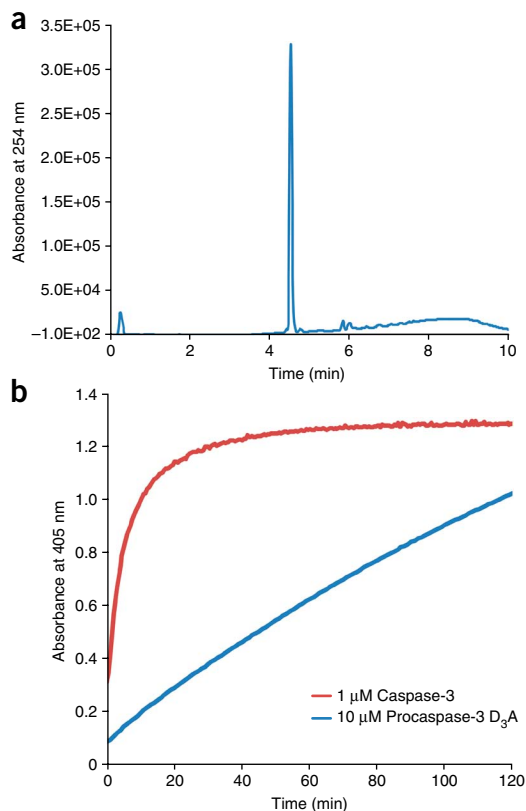
Steps 55–58, Global deprotection of Ac-DEVD-pNA: 20 h

### ? TROUBLESHOOTING

If the fully protected product is not pure after washing with diethyl ether, the product may be further washed with diethyl ether to remove further impurities. Alternatively, the product may be purified by reverse-phase HPLC using a C18 column and a gradient elution of 95:5  $\text{H}_2\text{O}$ :ACN with 0.1% formic acid to 5:95  $\text{H}_2\text{O}$ :ACN with 0.1% formic acid over 10 min.

### ANTICIPATED RESULTS

As described, the synthesis will produce 700–1,000 mg of Ac-DEVD-pNA. The procedure can be scaled if desired. The Ac-DEVD-pNA product may be evaluated as a substrate for procaspase-3, caspase-3, procaspase-7 or caspase-7 by a standard enzyme



**Figure 4 |** Evaluation of Ac-DEVD-pNA. **(a)** HPLC-trace (detection at 280 nm) for final product shows single peak at 4.58 min. Analysis was run on a C18 column using a gradient of 95:5  $\text{H}_2\text{O}$ :ACN with 0.1% formic acid to 5:95  $\text{H}_2\text{O}$ :ACN with 0.1% formic acid over 10 min. **(b)** Progress curves for the enzymatic hydrolysis of Ac-DEVD-pNA. A 1  $\mu\text{M}$  solution of caspase-3 and a 10  $\mu\text{M}$  solution of the uncleavable procaspase-3 ( $\text{D}_3\text{A}$ ) mutant<sup>2,36</sup> were incubated with 200 M of Ac-DEVD-pNA in a buffer containing 50 mM HEPES (pH 7.4) and 300 mM NaCl. The absorbance at 405 nm was read over the 2 h incubation.

activity assay. As shown in **Figure 4b**, when this substrate (at 200 μM in 50 mM Hepes (pH 7.4), 300 mM NaCl buffer) is treated with caspase-3 (1 μM final concentration) or an uncleavable procaspase-3 (D<sub>3</sub>A) mutant<sup>2,36</sup> (10 μM final concentration), an increase in absorbance at 405 nm is observed.

**Analytical data**

Ac-DEVD-pNA:

m.p.: 179–184 °C

HPLC (C18, See **Fig. 4a** for trace)

<sup>1</sup>H-NMR (500 MHz, CD<sub>3</sub>OD) (see **Supplementary Data**)

HRMS(ESI) (m/z) calculated for C<sub>26</sub>H<sub>34</sub>N<sub>6</sub>O<sub>13</sub>, 639.22; found, 639.2254

Note: Supplementary information is available via the HTML version of this article.

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