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Solid Phase Synthesis of Peptide Aldehyde Protease Inhibitors. Probing the Proteolytic Sites of Hepatitis C Virus Polyprotein

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Abstract: The solid phase synthesis of a set of peptide aldehydes derived from the NS5A/NS5B junction of hepatitis C virus (HCV) viral polyprotein is demonstrated using an oxazolidine linker and the Multipin™ method. Deletion of the P6 and P5 residues results in a dramatic loss of inhibitory activity. Copyright © 2000 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: hepatitis C virus (HCV); peptide aldehyde; protease inhibitors; solid phase peptide synthesis

INTRODUCTION

Infection by the hepatitis C virus (HCV) is a serious medical problem afflicting $>1\%$ of the human population [1–3]. Protective vaccines are not available and α-interferon, the most commonly approved therapy, is effective in fewer than 25% of the cases [4]. Individuals who become chronically infected have the potential to develop hepatitis and potentially fatal hepatocellular carcinoma. In a similar fashion to HIV, there is a desperate need to develop alternative therapies aimed at controlling viral replication and this has stimulated concerted efforts worldwide in the area of small molecule inhibitor design. HCV is an enveloped, positive-stranded RNA virus with a single stranded RNA genome that encodes a single polyprotein of 3010–3033 amino acids. The translated HCV polyprotein is proteolytically processed by a combination of host- and virus-encoded enzymes into nine distinct polypeptides: 5′-C-E1-E2-NS2-NS3-NS4A-NS4B-NS5A-NS5B-3′ [4]. Proteolysis at the NS3/NS4A, NS4A/NS4B, NS4B/NS5A and NS5A/NS5B sites is catalysed by the NS3 protein (non-structural protein 3). NS3 is a chymotrypsin-like serine protease with a characteristic Ser, His and Asp catalytic triad. Since the protease is likely to be essential for viral replication, it is an attractive target for inhibitor development. Although the goal is to develop a therapeutic non-peptidic protease inhibitor, critical structure/activity relationships at the NS5A/5B cleavage site can be elucidated by screening sets of peptide aldehydes as substrate-based inhibitor probes. Peptide aldehydes are useful substrate analogues for both serine and cysteine protease active sites. They form covalent tetrahedral adducts, hemiacetals (hemithioacetals with cysteine proteases), with the active site serine or cysteine, which specifically mimic the tetrahedral transition states of the enzyme reaction pathway [5].

Abbreviations: Ac_2O, acetic anhydride; AcOH, acetic acid; BSA, bovine serum albumin; DCM, dichloromethane; DIC, diisopropylcarbodiimide; DIEA, diisopropylethylamine; DMSO, dimethylsulfoxide; DTT, dithiothreitol; DMF, dimethylformamide; ESMS, electrospray mass spectrometry; Fmoc, 9-fluorenyl-methoxycarbonyl; HPLC, high performance liquid chromatography; HOBt, 1-hydroxybenzotriazole; MeCN, acetonitrile; MeOH, methanol; THF, tetrahydrofuran; TFA, trifluoroacetic acid.

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In order to adequately carry out this work, a simple method of synthesizing peptide aldehydes on the solid phase was required. A parallel synthesis methodology was desirable because multiple peptide aldehydes in a short time span were needed. In an earlier model study, a new linker was developed, which was stable to the conditions of Fmoc peptide synthesis, including TFA treatment, but is cleaved by mild aqueous acid at 60°C [6]. The mild cleavage conditions allow the straightforward synthesis of peptide aldehydes that are not contaminated with cleavage reagents, which may affect sensitive biological assays. The solid phase synthesis of peptide aldehydes via the oxazolidine linker [6] is just one of a number of reported methods, including the semicarbazone method [7], the Weinreb amide method [8], the ozonolysis method [9,10], the thiazolidine method [11], the backbone amide linker method [12] and oxidation of support-bound peptide alcohols [13]. The oxazolidine linker satisfies the following criteria for a general linker for the immobilization of aldehyde functionality: (i) it is cheap and easy to construct, (ii) it is chemically stable (long shelf life), (iii) it does not require preformation (i.e. the aldehyde couples directly to the solid phase), (iv) aldehyde attachment is generic, and (v) it cleaves under mild conditions which leave no residue upon evaporation. In this paper, the synthesis of peptide aldehydes homologous with the NS5A/NS5B junction is presented. These were used to elucidate critical binding information and can be used as a starting point for development of peptidomimetic inhibitors of viral replication.

MATERIALS AND METHODS

Peptides were synthesized on SynPhase™-MD-I Series crowns (loading 7.5 μmol/crown, code SPMDINOF), available from Chiron Technologies (Melbourne, Australia). These supports had been radiation grafted with methacrylic acid/dimethylacrylamide (MD) and functionalized with Fmoc-Gly. Fmoc-protected amino acids were used throughout. The following side-chain protected residues were used: Cys(Trt), Asp(*t*Bu) and Glu(*t*Bu).

Synthesis of the protected amino acid aldehydes was achieved via reduction of the corresponding *N*,*O*-dimethylhydroxamates with $LiAlH_4$ by adapting the method of Fehrentz and Castro [14]. The synthetic details of **2**, **3** and the *C*-terminal Abu peptide aldehyde **15a** illustrates the method.

Synthesis of Fmoc-Abu-NMeOMe 2

To a stirred solution of Fmoc-Abu-OH **1** (0.708 g, 2.18 mmol), *N*,*O*-dimethylhydroxyamine hydrochloride (0.215 g, 2.18 mmol) and benzotriazoyl-1-yl-oxy-tris-(dimethylamino)-phosphonium-hexafluorophosphate (0.986 g, 2.18 mmol) in dichloromethane (30 ml) in a round bottom flask, triethylamine (0.63 ml, 4.86 mmol) was added. The mixture was stirred at room temperature for 16 h. The resulting solution was washed successively with water (50 ml), 10% aqueous citric acid (50 ml), water (50 ml), 5% aqueous $NaHCO_3$ (50 ml), water (50 ml) and brine (50 ml). The organic extract was dried (Na_2SO_4) and the dichloromethane removed (reduced pressure) to give Fmoc-Abu-*N*,*O*-dimethylhydroxamate **2** as a clear oil (0.671 g, 84%). HPLC (purity = 95% at 214 nm) ESMS: found 369.1 $[M+H]^+$; expected 369.1 for $[C_{21}H_{24}N_2O_4+H]^+$. ^{1}H-NMR ($CDCl_3$) of **2**: δ 0.93 (t, $J=8$ Hz, 3H), 1.62 (m, 1H), 1.78 (m, 1H), 3.20 (s, 3H), 3.78 (s, 3H), 4.20 (t, $J=7$ Hz, 1H), 4.33 (m, 2H), 4.69 (m, 1H), 5.46 (d, $J=8$ Hz), 1H), 7.26–7.74 (m, 8H). EI-HRMS m/z 368.1738 (calcd. for $C_{21}H_{24}N_2O_4$ m/z 368.1736).

Synthesis of Fmoc-Abu-H 3

In a round bottom flask, under an atmosphere of nitrogen and at −78°C (dry ice, acetone bath) a slurry of lithium aluminium hydride (0.40 g, 10.5 mmol) was stirred in THF (5 ml). To this mixture, dropwise over 3 min, a solution of Fmoc-Abu-NMeOMe **2** (0.671 g, 1.83 mmol) in THF (5 ml) was added. The mixture was stirred for an additional 10 min after which diethyl ether (30 ml) was added and the mixture allowed to warm to room temperature. To this mixture 10% aqueous citric acid (20 ml) was added dropwise and the mixture stirred for 30 min. This mixture was extracted with diethyl ether (2 × 30 ml). The organic extract was washed with water (50 ml) and brine (50 ml). The organic extract was dried (Na_2SO_4) and evaporated (reduced pressure) to yield Fmoc-Abu-H **3** as an opaque waxy solid (0.584 g, 98%), which was used immediately for the next step (Fmoc amino acid aldehydes have limited stability but can be stored at −20°C) [14]. ^{1}H-NMR ($CDCl_3$) of **3**: δ 0.89 (t, $J=8$ Hz, 3H), 1.64 (m, 1H), 1.92 (m, 1H), 4.15 (t, $J=7$ Hz, 1H), 4.22 (m, 1H), 4.36 (d, $J=7$ Hz, 2H), 5.27 (d, $J=5$ Hz, 1H), 7.23–7.70 (m, 8H), 9.55 (s, 1H).

Preparation of Support-Bound Threonine Aldehyde Linker 4

To a glass vial (8 ml) ten SynPhase™ crowns (loading 7.5 μmol/crown) derivatized with Fmoc-Gly, were added. The Fmoc group was removed with 20% piperidine/DMF (5 ml) for 30 min. The crowns were washed (all washes with crowns were 5 min) with DMF (2 × 5 ml), MeOH (2 × 5 ml) and air dried. To the vial containing the crowns a solution of Fmoc-Thr (170 mg, 0.5 mmol), DIC (78 μl, 0.5 mmol) and HOBt (68 mg, 0.5 mmol) in DMF (5 ml), 0.1 M or ~6–7 equivalents, was added. The reaction was allowed to proceed for 2 h at room temperature after which the reaction mixture was drained of solution and the crowns were washed with DMF (2 × 5 ml). To the washed crowns a solution of 20% piperidine in DMF (5 ml) was added and the reaction allowed to proceed for 30 min at room temperature. The reaction mixture was drained of solution and the crowns were washed with DMF (3 × 5 ml), MeOH (2 × 5 ml) and air dried to yield crowns derivatized with threonine aldehyde linker **4**.

Synthesis of Support-Bound Fmoc-Abu-H Oxazolidine Aldehyde Linker 6

SynPhase™ crowns (10) **4** were placed into sealable glass vials (Wheaton, 20 ml, Millville, NJ). The aldehyde Fmoc-Abu-H **3** (0.292 g, 0.9 mmol) was dissolved in a 1% DIEA/MeOH solution (9 ml) to give a 0.1 M solution (~12 equivalents, but 3–4 is adequate). This solution was added to the crowns and the mixture heated at 60°C for 2 h. The reaction mixture was drained of solution and the crowns washed with MeOH (3 × 10 ml). The yield of attachment of Fmoc-Abu-H to the threonine linker can be determined spectrophotometrically by cleaving the Fmoc protecting group with 20% piperidine in DMF and measuring absorbance at 301 nm. A single crown was treated with 20% piperidine/DMF (10 ml) for 30 min at 20°C and the absorbance of the solution was determined at 301 nm after diluting the deprotection solution 1:10 in 20% piperidine/DMF, and using the following equation: Loading (nmol/crown) = (Abs/0.0078) × 110. The loading was determined to be 90% (6.7 μmol).

Synthesis of Ac-Glu-Asp-Val-Val-Abu-Abu-H 15a

Fmoc-Abu-H **3** was reacted with ten derivatized SynPhase™ crowns **4** to give **6** as described above. The crowns were then subjected to Fmoc-peptide synthesis using the following conditions. (i) Fmoc deprotection: 20% piperidine in DMF (10 ml) for 30 min, followed by washing with DMF (3 × 10 ml) and DCM (2 × 10 ml). (ii) Coupling conditions: Fmoc protected amino acid (1 mmol), DIC (1 mmol), HOBt (1 mmol) in DMF (10 ml), 2 h at 20°C. Following all couplings, the crowns were washed with DMF (2 × 10 ml), DCM (2 × 10 ml) and air dried. Acetylation of the *N*-terminus was achieved with Ac_2O:DIEA:DMF (ratio 3:1:96 v:v:v) at 4°C for 5 min only. The crowns were washed with DMF (2 × 10 ml), DCM (2 × 10 ml) and air dried. (iii) Side-chain deprotection and cleavage: each crown was treated with TFA (2.5 ml per crown) for 30 min after which the crowns were washed with DCM (2 × 2 min) and air dried. The peptide aldehyde **15a** was cleaved with TFA:MeCN:H_2O (0.1:60:40, v:v:v) (2.5 ml per crown) at 60°C for 30 min. Each crown was removed and the cleavage solution lyophilized to give **15a** as a white solid (3.5 mg from one crown, 79%). HPLC (purity = 95% at 214 nm). See Table 1 for analytical data. HPLC purification yielded pure stereoisomers **15b** and **17** (see Figure 1).

Analysis and Purification of Compounds 2, 3, 8–17

Analytical HPLC was performed on a Waters chromatography system using a Ranin microsorb-mv (#86-200-F3) RP-18 column (100 A, 3 μm). The following condition was used: buffer A = water (0.1% H_3PO_4); buffer B = 90% MeCN/10% water (0.1% H_3PO_4); linear gradient A to B from 1 to 11 min; flow rate = 1.5 ml/min. Absorbances were recorded at 214 and 254 nm. HPLC purities were determined by peak area at 214 nm. Reverse phase HPLC purification was carried out using a Waters Maxima 820 RP-HPLC system linked to a Vydac 218TP1010 C18 (1.0 × 25 cm) column at a flow rate of 4 ml/min. The following condition was used: buffer A = water (0.1% TFA); buffer B = 90% MeCN/10% water (0.1% TFA); linear gradient A to B from 3 to 27 min.

^{1}H-NMR spectra were recorded on a 400 MHz Varian ^{UNITY}INOVA spectrometer using $CDCl_3$ or DMSO as solvent. LCMS analyses were performed on a Perkin Elmer Sciex API III mass spectrometer linked to a Shimadzu LC-10AD HPLC system. The following condition was used: the column was a Monitor 5 μm C18 50 × 4.6 mm (Column Engineering, CA), buffer A = water (0.1% TFA); buffer B = 90% MeCN/10% water (0.1% TFA); linear gradient A to B from 0.5 to 11.5 min at a flow rate of 1.5 ml/min. Absorbances were recorded at 214 and 254 nm. The flow rate to the mass spectrometer was 300

Table 1 Characterization Data and HCV Protease Inhibitory Activity for Peptide Aldehydes **8**–**17**

Entry	Peptide aldehyde sequence	RP-HPLC data[a]		ESMS data		Inhibition of HCV protease
		% Purity (214 nm)	R_t (min)	Calculated $[M+H]^+$	Observed $[M+H]^+$	IC_{50} (μM)
8	Ac-Val-Val-Abu-Cys-H	40	6.38br	431.6	431.4	No activity[c]
9	Ac-Val-Val-Abu-Abu-H	94	6.50br	413.5	413.2	No activity[c]
10	Ac-Val-Val-Abu-Nva-H	93	7.00br	427.6	427.4	No activity[c]
11	Ac-Asp-Val-Val-Abu-Cys-H	59	6.30br	546.7	546.4	100–150
12	Ac-Asp-Val-Val-Abu-Abu-H	50	6.47br	528.6	528.4	100–150
13	Ac-Asp-Val-Val-Abu-Nva-H	65	6.87br	542.6	542.2	100
14	Ac-Glu-Asp-Val-Val-Abu-Cys-H	65	6.50br	675.8	675.4	20–30
15a	Ac-Glu-Asp-Val-Val-Abu-Abu-H	95	6.43br	657.7	657.4	30–40
16a	Ac-Glu-Asp-Val-Val-Abu-Nva-H	70	6.89br	671.8	671.4	10–12
15b	Ac-Glu-Asp-Val-Val-Abu-Abu-H	98[b]	6.43br	657.7	657.4	5.5
16b	Ac-Glu-Asp-Val-Val-Abu-Nva-H	98[b]	6.80br	671.8	671.4	12.4
17	Ac-Glu-Asp-Val-Val-Abu-D-Abu-H	>95[b]	6.51br	657.7	657.3	No activity[c]

[a] All products partially racemized at the *C*-terminus, with the exception of **15b** and **17**.
[b] Purified by HPLC.
[c] No inhibition at 100 μM.

μl/min after been split from the column (1.5 ml/min).

PROTEASE ASSAY

Spectrophotometric assays were run in a 96-well microtiter plate at 37°C using a SpectraMax 250 reader (Molecular Devices, Sunnyvale, CA) with kinetic capability. Substrate stock solution was prepared by dissolving Ac-Glu-Glu-Val-Val-Ala-Cys-pNA (Bachem, Switzerland) substrate (150 μM) in DMSO/DTT. Enzyme stock solution was prepared in 50 mM Hepes, 50% glycerol, 3 mM $MgCl_2$, 1 mM DTT, 0.1% BSA, pH 7.6 with an NS3 enzyme concentration of 150 nM. The test solution was obtained by adding a solution of peptide aldehyde in DMSO (5 μl) to the enzyme stock solution (85 μl). After incubation for 30 min at 20°C and then 30 min at 37°C, substrate stock solution (10 μl) was added and the *p*-nitroaniline absorbance was determined at 405 nm. The samples were read either continuously for 30 min or at 1 h.

RESULTS AND DISCUSSION

Peptide aldehyde synthesis using the oxazolidine linker requires pre-formation of individual protected amino acid aldehydes in solution (see Scheme 1). Synthesis of the protected amino acid aldehydes was achieved via reduction of the corresponding *N*,*O*-dimethylhydroxamates with $LiAlH_4$ by adapting the method of Fehrentz and Castro [14]. Reduction proceeds without racemization [14]. Reduction of the *N*,*O*-dimethylhydroxamates with $LiAlH_4$ proceeds through a stable lithium-chelated intermediate; further reduction of the lithium salt is precluded by intramolecular complexation. The aldehyde is then liberated upon aqueous work-up. *N*-Protected amino acid aldehydes are unstable, hence each was immediately condensed with Thr derivatized crowns **4** to form support-bound oxazolidine systems **6** (Scheme 2). The yield for attachment of Fmoc-Cys(Trt)-H, Fmoc-Nva-H and Fmoc-Abu-H was >90%. The loading and yield of derivatization was determined by quantitative Fmoc test at 301 nm ($\varepsilon = 7800$ per M/cm). In addition, the integrity of the attached aldehydes was determined by cleaving **6** and analysing the recovered Fmoc protected aldehyde **5** by HPLC.

The oxazolidine **6** is stable to non-aqueous acid and to base, and since the secondary amine is difficult to acylate, it can tolerate peptide synthesis conditions. Being stable to non-aqueous acid, trifluoroacetic acid (TFA) may be used to remove acid-labile protecting groups prior to release of the aldehyde function (i.e. cleavage). Standard peptide synthesis methodologies were used for the synthesis of all peptide aldehydes.

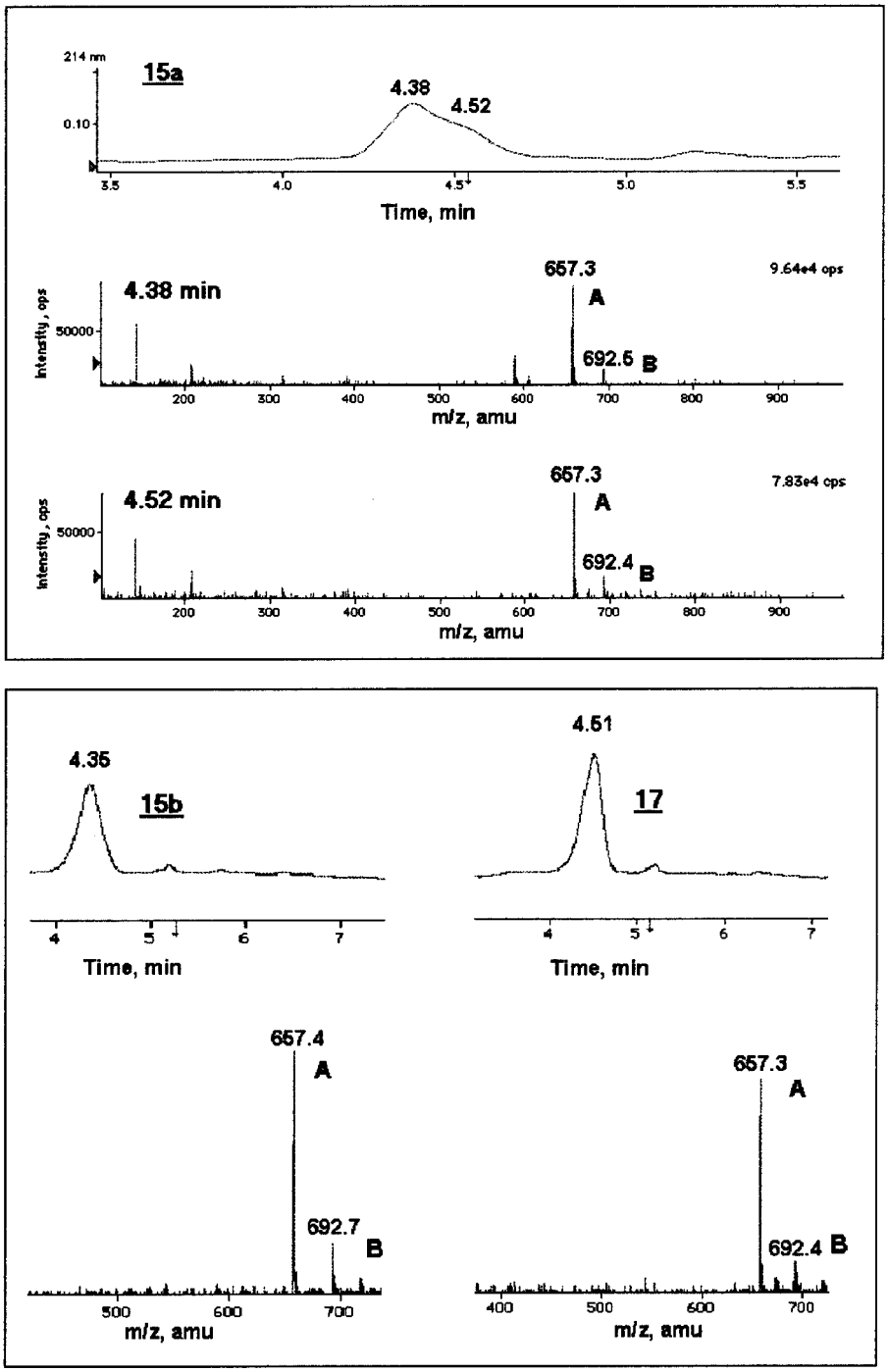
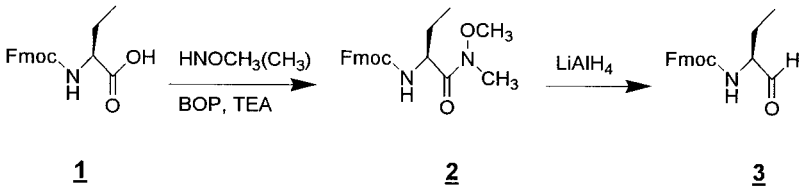


Figure 1 LCMS analysis of crude peptide aldehyde **15a** (top panel) and purified peptide aldehyde **15b**, L-Abu isomer, and **17**, D-Abu isomer (bottom panel). **A**: $[M+H]^+$, **B**: $[M+H+H_2O+NH]^+$.

Although there is potential to acylate the oxazolidine nitrogen during peptide synthesis, it is relatively unreactive [6,15] and no acylation occurs with DIC/HOBt activation. Acetylation with acetic anhydride is performed at 4°C for 5 min to avoid reaction with the oxazolidine nitrogen. Earlier studies with the oxazolidine linker showed cleavage fails to occur if the oxazolidine nitrogen is acetylated [6].

The support-bound peptide **7** was cleaved from the solid phase to yield peptide aldehydes **8**–**17** using a

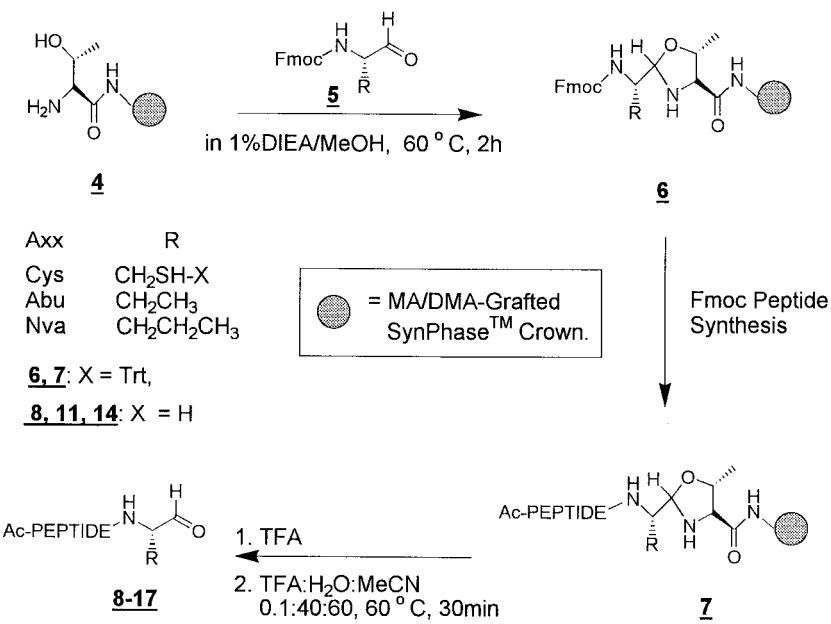


Scheme 1 Synthesis of protected amino acid aldehydes.

two-stage cleavage protocol involving side-chain deprotection with neat TFA followed by mild cleavage with 0.1% TFA in MeCN:water (60:40 v:v). The two-stage cleavage system is advantageous because complete side-chain deprotection can be performed, and the by-products are removed by washing before the peptide aldehyde is cleaved from the solid support. The mild cleavage conditions allowed for direct testing of unpurified peptide aldehydes **8**–**17** in a chromogenic protease assay. The analytical data for peptide aldehydes **8**–**17** are presented in Table 1.

LCMS and ^{1}H-NMR analysis of the peptide aldehydes indicated that the *C*-terminal residue was partially racemized, presumably on cleavage. The RP-HPLC traces of all peptide aldehydes show broad peaks. This is due primarily to the aldehyde being in equilibrium with the hydrated form under aqueous conditions [5]. The degree of racemization is sequence dependent, but was found to be greatest for *C*-terminal α-amino-butyraldehydes (i.e. Abu-H). LCMS analysis of peptide aldehyde **15a** indicated approximately 40% racemization, with the D-Abu isomer **17** eluting after the L-Abu isomer **15a** (see Figure 1). The diastereoisomers were separated during purification to yield purified **15b** and **17**. The ^{1}H-NMR spectrum of **15b** is presented in Figure 2. The aldehyde proton signal is clearly visible at 9.4 ppm. The purified peptide aldehyde **15b** was used to obtain an accurate IC_{50} against NS3 protease of 5.5 μM. The D-Abu diastereoisomer **17** is completely inactive against NS3 protease. The presence of racemization is unfortunate since it is desirable to screen diastereomerically pure compounds. In initial screening, a degree of racemization may be tolerable, although purification is advisable for detailed SAR studies.

The utility of solid phase peptide aldehyde synthesis using the oxazolidine linker was demonstrated by the synthesis of a small set of peptide aldehydes, which was designed to probe the relative importance of P-side residues to enzyme inhibition. As presented in Table 1, we found Cys (P1 in native



Scheme 2 Solid phase synthesis of peptide aldehydes.

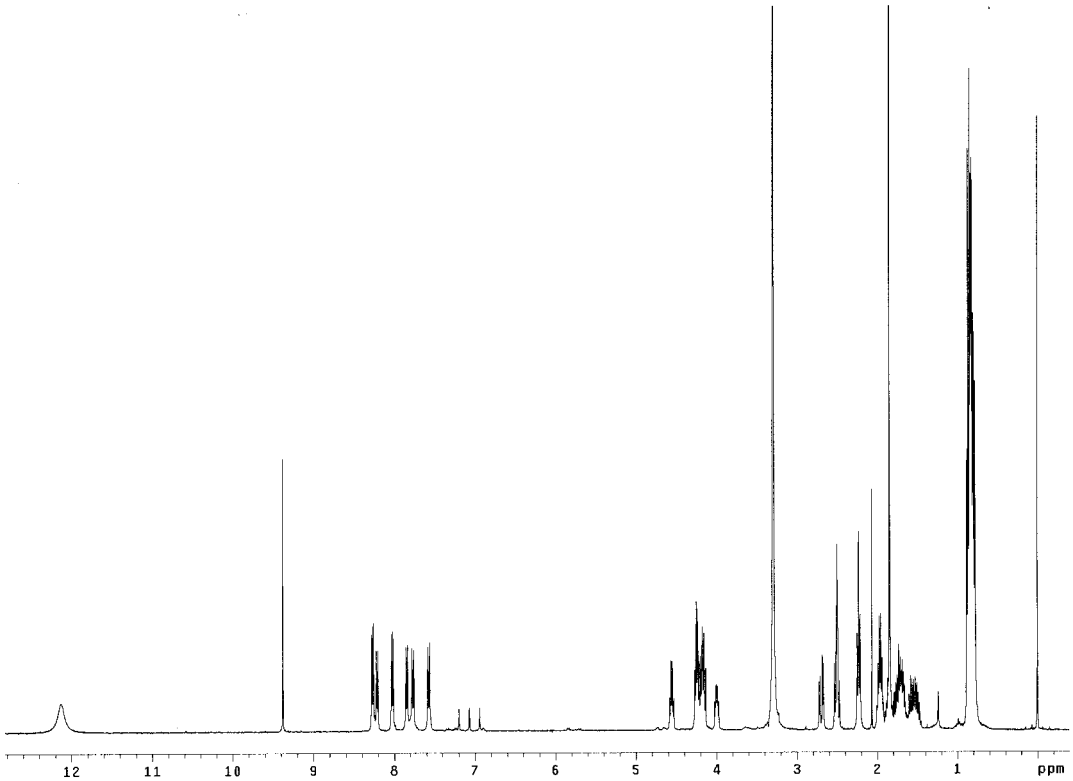


Figure 2 ^{1}H-NMR spectrum of purified peptide aldehyde **15b**. Note: side-chain carboxyl protons: 12.1 ppm, aldehyde proton: 9.4 ppm, amide NH protons: 7.5–8.3 ppm, α-CH protons: 4.0–4.6 ppm, H_2O: 3.3 ppm, DMSO (obscured) 2.49 ppm.

NS5A/NS5B junction) could be replaced by small hydrophobic residues, such as α-aminobutyric acid (Abu) and norvaline (Nva). Recent reports have described results consistent with these P1 substitution studies, using peptide acid inhibitors [16,18]. Interestingly, in what is an unusual observation for serine proteases [16], Ingalinelli and co-workers found that the *C*-terminal carboxyl peptides, corresponding to products resulting from the cleavage of the NS4A-NS4B and NS5A-NS5B peptide substrate, act as competitive inhibitors of NS3 protease. Analogous *C*-terminal carboxamide and reduced alcohol sequences, however, are completely inactive [16]. A further consistent finding between the aldehyde set **8**–**17** (Table 1) and the reported peptide acids was that P6 and P5/P6 truncation sequences derived from the NS5A/NS5B junction were poor inhibitors [16–18]. Interestingly, the carboxylate function at P6 is as critical to binding as the functionality at the *C*-terminus, which can be either a carboxylate or an aldehyde moiety, but not an amide or alcohol moiety.

CONCLUSION

Sets of peptide aldehydes, prepared by the straightforward approach described here, were demonstrated to be effective tools in expediting rapid SAR analysis of serine proteases, such as NS3 protease.

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