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Progress in the preparation of peptide aldehydes via polymer supported IBX oxidation and scavenging by threonyl resin‡

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Abstract: Peptide aldehydes are of interest due to their inhibitory properties toward numerous classes of proteolytic enzymes such as caspases or the proteasome. A novel access to peptide aldehydes is described using a combination of solid phase peptide synthesis with polymer-assisted solution phase synthesis based on the oxidation of peptide alcohols with a mild and selective polymer-bound IBX derivative. The oxidation is followed by selective purification via scavenging the peptide aldehyde in a capture-release procedure using threonine attached to an aminomethyl resin. Peptide aldehydes are obtained in excellent purity and satisfying yield. The optical integrity of the *C*-terminal residue is conserved in a high degree. The procedures are compatible with the use of common side-chain protecting groups. The potential for using the method in parallel approaches is very advantageous. A small collection of new and known peptide aldehydes has been tested for inhibitory activity against caspases 1 and 3.

Keywords: polymer-assisted synthesis; polymer-bound reagents; peptide aldehydes; caspase inhibitors; oxidation; IBX; fluorescence assay

INTRODUCTION

Peptide aldehydes are generally either cysteine- or serine-protease inhibitors. Several natural peptide aldehydes have been isolated from *Streptomyces* species [1]. Examples are leupeptine [2], chymostatine, antipaine, elastinal and β-MAPI [3]. The selectivity of peptide aldehydes is a problem since they generally inhibit both serine- and cysteine-proteases. A certain degree of selectivity can be achieved by altering the peptide sequence. Therefore the substrate specificity of the enzyme along P′ with *C*-terminal peptide aldehydes can not be considered. Numerous applications have been reported for the inhibition of the proteasome or the caspases [4]. Caspases (cysteine-dependent, aspartic acid-directed proteases) are cysteine-proteases [5] with a high specificity towards aspartate in P1 of their substrate. They play an important role as effectors in inflammatory diseases (caspases 1, 4 and 5) as well as in apoptosis (caspases 2, 3, 6, 7, 8, 9 and 10) [5–7]. Numerous methods for the synthesis of peptide aldehydes have been described, involving the reduction of carboxylic acid derivatives or oxidation of the corresponding alcohol. Both approaches have been performed with great variety, in both solution-phase syntheses [8–11] and solid-phase syntheses [12–20].

Despite these efforts, no generally applicable method with a reliable and uncomplicated protocol has been found so far. The parallel synthesis of peptide aldehyde collections for screening in biological assays is still a serious problem, especially when using trifunctional amino acids.

The key problem of the synthetic process is the oxidation of the amino alcohol functionality, whereas the peptide synthesis involves fewer problems even for peptidomimetics. Previously reported solution phase methods use DMSO-mediated oxidation procedures [8,9] such as the Parikh-Doering oxidation [21]. The preceding reduction of amino acid derivatives to the corresponding amino alcohols is accomplished with reagents such as $LiBH_4$ or $NaBH_4$. Other approaches to preparing amino alcohols are the Soai reduction of amino acid anhydrides [22], the pentafluorophenol active ester method [23], and the use of mixed anhydrides from ethyl chloroformate [24,25].

Synthesis of amino aldehydes without prior reduction to the alcohol and a subsequent oxidation step were accomplished with diisobutylaluminium hydride (DIBAH) [25] including the risk of over-reduction. Synthesis via Weinreb amides [10] provides a more suitable method but with the disadvantage of cleaving ester side-chain protecting groups during the reduction step [26]. All aldehydes prepared by these methods have to be purified either by crystallization or chromatography. Chromatography often affects peptide epimerization of the α-carbon atom at the *C*-terminal amino aldehyde to a high degree [25]. Some improvements are feasible but they are laborious [25,27].

Several approaches for peptide aldehyde synthesis on solid supports are described. The most popular builds up the peptide from the polymer-bound amino acid

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‡ Dedicated in memory to our dear colleague Murray Goodman.

on ester or amide linkers, followed by direct reductive cleavage to the corresponding peptide aldehyde. Especially the reduction of immobilized esters, mainly of phenyl esters, forming the aldehyde often proceeds with overreduction [13,14]. Resin-attached Weinreb amides promise better results, although the literature describes mainly the synthesis of simpler peptide aldehydes containing aliphatic side chains. Another difficulty is the dependency of the applied amount of $LiALH_4$ on the peptide chain length, which diminishes the general applicability to parallel approaches. Recently a synthesis of peptide aspartyl aldehydes was published, however, with low yields and the need for chromatographic purification to remove toxic $LiAlH_4$ by-products [15]. The reduction of Weinreb amides occurs without racemization, but this advantage is lost during the purification.

Another approach uses urethane-protected amino aldehydes synthesized in solution and resin attachment is performed after peptide synthesis. The precursors often require considerable synthetic effort, because the linker has to be attached to the precursor before immobilization. Examples are the syntheses via semicarbazones [16], thiazolidines [17] or acetals [18].

A semicarbazone linker for direct attachment of aldehydes to a solid support has been described [19]. These semicarbazones are more stable than peptide aldehydes and can be cleaved from solid supports without racemization. The subsequent release of the aldehydes with pyruvic acid is racemization-free as well. However, chromatographic purification is necessary. An elegant peptide aldehyde synthesis suitable for simple peptides with almost no by-product formation is the solid phase synthesis of peptide olefins followed by ozonolysis [28]. Product purities of 90% determined by HPLC are reported, but no information about the purities of the diastereomers is given. A very gentle approach is the *C*-terminal attachment of amino aldehydes as oxazolidines with the use of serine or threonine linkers on 'Synphase Crowns' [20], under weakly basic anhydrous conditions at 60 °C.

The following reports a novel combination of three procedures on solid phase: peptide alcohol synthesis, oxidation with a polymer-bound reagent and purification with a scavenger-release polymer. The criteria in the choice of peptide aldehyde sequences to illustrate our technology are the biological relevance as well as the chemical diversity. Therefore some known biologically important inhibitors have been included and tested for their inhibition efficiency against caspases 1 and 3. To determine the applicability of the synthesis and capture-release procedures for different *C*-terminal amino acids, the peptide sequence of FVAX-H was kept constant and the amino alcohol X varied.

RESULTS AND DISCUSSION

Our aim was to develop a robust and routine procedure for the preparation of peptide aldehydes as caspase inhibitors which would be applicable for the incorporation of protected trifunctional amino acids. In contrast to most of the previously described methods [10–21], this approach is suitable for both parallel and automated technologies [29,30].

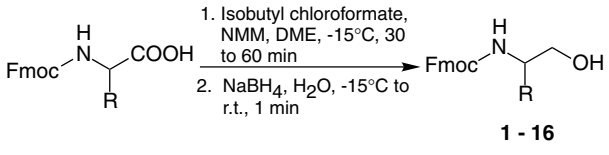
An indirect approach was used via SPPS of the N^{α}-Fmoc-protected peptide alcohols. First the corresponding N^{α}-Fmoc and side-chain protected amino alcohols were prepared and attached to the TCP-resin. After peptide synthesis and cleavage from the TCP-resin, the IBX-resin oxidation step was accomplished in solution [31]. The crude products were purified with a novel capture-release procedure on the threonyl resin and analysed by HPLC, MS and NMR.

Preparation of Fmoc-β-Amino Alcohols from Fmoc-α-Amino Acids

Carboxy groups have been activated for reduction as active esters [32,33], acid fluorides [34], N-carboxy anhydrides [35] and mixed anhydrides using isobutyl chloroformate [24,36]. The latter has proven to be the best procedure with respect to handling, costs and yields. The reduction proceeds rapidly with $NaBH_4$ at room temperature and tolerates amides, carbamates and unactivated carboxylic esters as side-chain functions (Scheme 1). The Fmoc-amino alcohols **1–16** (Table 1) were prepared on a 25 mmol scale. Some of the Fmoc-amino alcohols had to be purified by column chromatography. The purities after flash-column chromatography were about 96%, and the yields ranged from 25% to 94%. Analysis of the Fmoc-amino alcohols **1–16** (Table 1) was accomplished by HPLC, ESI-MS and ^{13}C-NMR.

Synthesis of Peptide Alcohols

The peptide aldehydes were synthesized in four steps on the TCP-resin which carries (4-chlorodiphenyl-methyl)benzoic acid attached to aminomethyl polystyrene resin cross-linked with 1% divinylbenzene (capacity: 1.0 mmol g^{-1}). The Fmoc-amino alcohol was attached to the TCP-resin by substitution of the chloride using pyridine or DIEA as base (Scheme 2). The loadings ranged from 0.4 to 0.6 mmol g^{-1} as

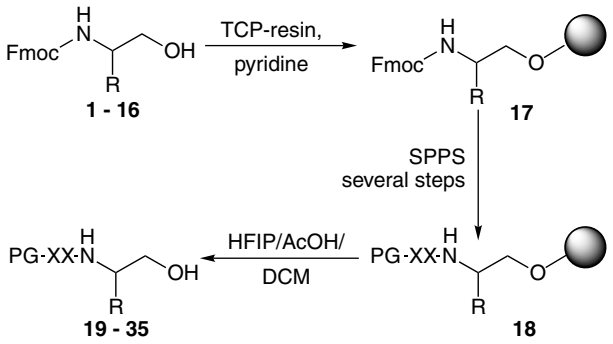


Scheme 1 Synthesis of β-amino alcohols (**1–16**) from α-amino acids [24].

Table 1 Synthesis of Amino Alcohols

Amino alcohol	Yield (purified)	Purity[a] [HPLC]	ES-MS $[M + H]^+$
Fmoc-Cys(Trt)-ol (**1**)	51% (7.3 g)	>99%[a]	572.2
Fmoc-Asp(tBu)-ol (**2**)	72% (7.2 g)	>99%[a]	398.0
Fmoc-Glu(tBu)-ol (**3**)	85% (8.7 g)	TLC pure	412.0
Fmoc-Phe-ol (**4**)	92% (8.6 g)	>99%	374.2
Fmoc-Gly-ol (**5**)	94% (7.0 g)	>99%[a]	284.1
Fmoc-His(Trt)-ol (**6**)	62% (9.6 g)	pure (TLC)	606.2
Fmoc-Ile-ol (**7**)	78% (6.9 g)	pure (TLC)	340.1
Fmoc-Lys(Boc)-ol (**8**)	80% (9.4 g)	>99%[a]	455.2
Fmoc-Leu-ol (**9**)	73% (6.2 g)	>99%[a]	340.0
Fmoc-Met-ol (**10**)	95% (8.5 g)	97%[a]	358.0
Fmoc-Asn(Trt)-ol (**11**)	25% (3.6 g)	>99%[a]	583.3
Fmoc-Arg(Pbf)-ol (**12**)	62% (9.8 g)	96%[a]	635.7
Fmoc-Ser(tBu)-ol (**13**)	59% (5.7 g)	pure (TLC)	370.0
Fmoc-Val-ol (**14**)	70% (6 g)	pure (TLC)	326.0
Fmoc-Trp(Boc)-ol (**15**)	67% (8.8 g)	>99%[a]	513.2
Fmoc-Tyr(tBu)-ol (**16**)	75% (8.3 g)	pure (TLC)	446.1

[a] After purification.



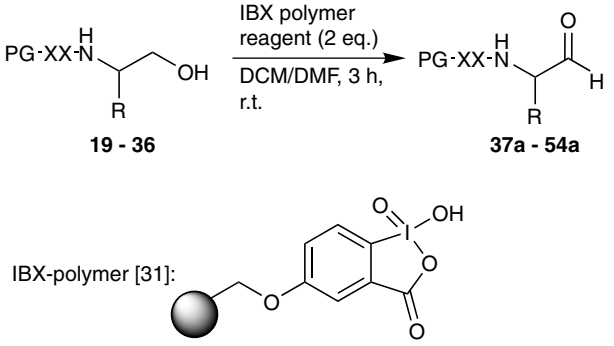
Scheme 2 Solid phase synthesis of peptide alcohols (**19–36**) PG = protecting group Ac or Z.

determined by UV absorption of the cleaved Fmoc-protecting group products. The synthesis of the peptide sequence was performed using dichloromethane (DCM) and dimethylformamide (DMF) as solvents and DIC-HOBt as coupling reagent. The success of the coupling steps was determined qualitatively with the 'Kaiser test' [37]. The *N*-terminal amino groups were acetylated or protected by the benzyloxycarbonyl group. Cleavage from the resin was accomplished using a mixture of hexafluoroisopropanol (HFIP) and acetic acid in DCM (Scheme 2). The reactions were performed on a 120 µmol scale. The peptide syntheses were carried out either manually or using a simultaneous multiple peptide synthesizer. The purities and identities of the *N*-terminally blocked and side-chain protected peptide alcohols **19–36** were determined by HPLC, ESI-MS and ^{1}H-NMR (Table 2).

Table 2 Side-chain Protected Peptide Alcohols. Protecting Groups as shown in Table 1

Peptide alcohol	Yield	Purity [HPLC]	ES-MS $[M + H]^+$
Ac-LLL-ol (**19**)	70% (32.5 mg)	97%	386
Ac-FVAV-ol (**20**)	72% (40.0 mg)	99%	463
Z-LLV-ol (**21**)	88% (49.0 mg)	97%	464
Ac-FVAG-ol (**22**)	58% (29.0 mg)	99%	421
Ac-FIW-ol (**23**)	49% (34.6 mg)	97%	593
Ac-WEHD-ol (**24**)	30% (37.9 mg)	91%	1068
Ac-YVAD-ol (**25**)	46% (33.5 mg)	98%	607
Z-GFG-ol (**26**)	63% (30.0 mg)	>99%	400
Z-IEAL-ol (**27**)	81% (60.0 mg)	98%	621
Z-LLI-ol (**28**)	75% (43.0 mg)	98%	478
Ac-FVAL-ol (**29**)	61% (35.0 mg)	98%	477
Ac-FVAI-ol (**30**)	91% (52.0 mg)	97%	477
Ac-FVAS-ol (**31**)	71% (43.0 mg)	99%	507
Ac-FVAD-ol (**32**)	50% (32.0 mg)	97%	535
Ac-A-Thz-D-ol[a] (**33**)	70% (36.0 mg)	99%	429
Ac-FVAK-ol (**34**)	65% (46.0 mg)	97%	592
Ac-LVAM-ol (**35**)	55% (33.0 mg)	96%	495
Ac-LLM-ol (**36**)	52% (25.0 mg)	93%	404

[a] Thz = 4-Aminomethyl-thiazole-2-carboxylic acid [38].



Scheme 3 Polymer-assisted solution phase (PASP) synthesis of peptide aldehydes (**37a–54a**) with the oxidizing IBX polymer reagent [31].

Oxidation of Peptide Alcohols

The solubility of the peptide alcohols **19–36** (Table 2) is best in methanol. However, there were two reasons to avoid this solvent. Firstly, methanol is oxidized by the IBX-resin and deactivates the polymer-reagent in this way. Secondly, the swelling of the polystyrene resin is poor in methanol. The peptide alcohols **19–36** were dissolved in mixtures of DCM and DMF, if necessary DMSO was added. It was also important to keep the reaction volume as small as possible for the oxidation procedure (Scheme 3). The IBX-resin was from a previously prepared batch with an oxidation capacity [31] of 0.71 mmol g^{-1}. After the oxidation the resin was filtered off and washed. The filtrate solvents

were removed under reduced pressure or in a nitrogen stream.

Scavenging Procedure

The crude peptide aldehydes prepared by various published procedures often contain considerable amounts of the corresponding peptide alcohols, as found by HPLC-MS (Figure 1). As an example, Ac-Val-Thz-Asp(OtBu)-H and the corresponding alcohol are shown, coeluting in a wide range of the chromatogram (Thz = 4-aminomethyl-thiazole-2-carboxylic acid as described by Videnov *et al.* [38]). Due to this alcohol content peptide aldehydes can not be purified by preparative HPLC. Possibly a semi-acetal is formed from peptide aldehyde and peptide alcohol which is in equilibrium with the monomers. The experiment shown in Figure 1 was an early attempt to oxidize a peptide alcohol with pyridine-

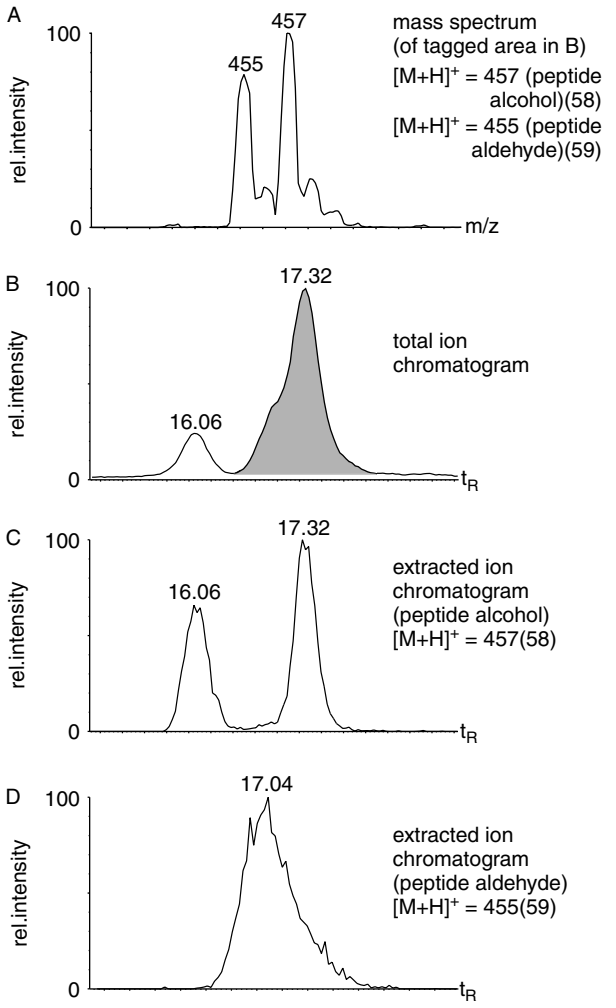


Figure 1 HPLC-MS of crude peptidomimetic aldehyde Ac-Val-Thz-Asp(OtBu)-H (**59**) after DMSO-mediated oxidation; A: TIC; B: mass trace of the alcohol (**58**); C: mass trace of the aldehyde (**58**); D: mass spectrum of the coeluted alcohol (**58**) and aldehyde (**59**). The crude product contains about 50% alcohol.

Table 3 Peptide Aldehydes Purified by Catch and Release Procedure

Peptide aldehyde	Yield	Purity [HPLC]	ES-MS $[M+H]^+$
Ac-LLL-H (**37b**)	49% (22.5 mg)	>99%	384
Ac-FVAV-H (**38b**)	36% (20 mg)	>99%	461
Z-LLV-H (**39b**)	25% (14.0 mg)	>99%	462
Ac-FVAG-H (**40b**)	30% (15.0 mg)	90%	419
Ac-FIW-H (**41b**)	8% (5 mg)	65%	493
Ac-WEHD-H (**42b**)	3% (2.3 mg)	93%	612
Ac-YVAD-H (**43b**)	6% (3.9 mg)	86%	493
Z-GFG-H (**44b**)	27% (13.0 mg)	>99%	398
Z-IEAL-H[a] (**45b**)	4% (3.0 mg)	85%	619
Z-LLI-H (**46b**)	21% (12.0 mg)	99%	476
Ac-FVAL-H (**47b**)	18% (10.0 mg)	96%	475
Ac-FVAI-H (**48b**)	23% (13.0 mg)	98%	475
Ac-FVAS-H (**49b**)	6% (3.5 mg)	70%	449
Ac-FVAD-H (**50b**)	10% (6.0 mg)	>99%	477
Ac-A-Thz-D-H (**51b**)	19% (8.4 mg)	96%	371
Ac-FVAK-H (**52b**)	19% (11.3 mg)	88%	490
Ac-LVAM-H (**53b**)	12% (6.0 mg)	92%	418[b]
Ac-LLM-H (**54b**)	7% (4.3 mg)	70%	509[b]

[a] Z-IEAL-H = Z-Ile-Glu(tBu)-Ala-Leu-H (**45b**) scavenged without cleavage of side-chain protecting groups.
[b] Sulfide oxidized by air oxygen.

SO_3·DMSO complex. Such conditions often lead to low purities of about 50% (Figure 1).

Ede *et al.* [20] modified 'Synphase Crowns' with a threonine linker to immobilize amino aldehydes for SPPS. This methodology was applied in reverse. A threonyl-resin should scavenge selectively the desired peptide aldehydes from the crude mixture after oxidation. In this way peptide aldehydes can be purified and separated from their peptide alcohols and other impurities. This new and generally applicable concept avoids the often unsuccessful attempts to separate alcohol and aldehyde by HPLC (Table 3).

Capture-Release Procedure

Aminomethyl-polystyrene resin (cross-linked with 1% divinylbenzene) was loaded with Fmoc-Thr(tBu)-OH. After deprotection with piperidine and then trifluoroacetic acid (TFA) the threonine loading was about 2 mmol g^{-1}. To scavenge the peptide aldehydes the threonyl resin (**55**) was treated with the crude oxidation products (**37a–54a**) in a mixture of water-free methanol, DMF, DCM (approximately 30 : 3 : 2, v : v : v; the exact ratio differed according to the solubility of the compounds) and 1% acetic acid. The reaction was allowed to proceed for 1 h at ambient temperature. The yields were found to be higher when working at a temperature of 50° to 60 °C, then, however, a high level of epimerized aldehyde was observed (Figure 3). The

procedure had to be carried out under anhydrous conditions to prevent cleavage of the scavenged aldehyde. The scavenging procedure was repeated if aldehyde was observed in the filtrate (Scheme 4).

For the aldehyde release two different routes were applied. Peptide aldehydes without side-chain protecting groups could be cleaved directly to yield the purified aldehyde. This was accomplished with a mixture of acetic acid, water, DCM and methanol (10 : 80 : 5 : 5; v : v : v : v). The procedure was repeated three times for 20 min at ambient temperature. Side-chain protecting groups containing peptide aldehydes were deprotected while still bound to the threonyl-resin. Boc or *tert.*-butyl protecting groups were cleaved with TFA, water and DCM (90 : 5 : 5; v : v : v). The filtrate from the cleavage mixture was analysed using HPLC and MS: no aldehyde, or only traces, could be detected. Trityl protecting groups were cleaved with TFA, water and triisopropylsilane (TIS) (95 : 2.5 : 2.5; v : v : v). Release of the deprotected aldehydes was performed as described above for unprotected aldehydes (Scheme 4).

The detachment of the peptide aldehydes involves a ring-opening stage. The rate of ring-opening and thus liberation of the aldehyde is strongly dependent on the acid/solvent system. For example, threonine oxazolidines derived from acetone or formaldehyde are cleaved with 95% TFA/water or 5% TFA/DCM [39], whereas benzaldehyde derivatives give less than 10% cleavage of the oxazolidines under these conditions. In contrast, treatment with AcOH/water at 60 °C for 30 min gives 100% cleavage. No cleavage occurs at 25 °C using acetic acid concentrations of 5%, 15%, 25% or 50% [20]. The relative stability of oxazolidines to TFA mixtures allows the deprotection of side-chain protecting groups of peptide aldehydes before cleavage from the solid phase.

The purities of the peptide aldehydes ranged from 65% to 99% (Table 3). One peptide aldehyde, Z-IEAL-H (**45b**), was cleaved without *tert.*-butyl ester deprotection. The methionine containing peptide aldehydes Ac-LVAM-H (**53b**) and Ac-LLM-H (**54b**) showed signals at $M^+ + 16$ amu in the mass spectra, showing that the sulfoxides had been formed — by air oxidation, because several treatments of sulfide-containing compounds with the IBX-resin revealed no *S*-oxidation. Sulfoxides can be reduced easily as described previously [40].

The applicability of our new capture-release strategy is best illustrated by the synthesis of the multifunctional caspase 1 inhibitor Ac-WEHD-H. The tetrapeptide carries four acid labile protecting groups namely Boc-, two *tert.*-butyl- and a trityl-group, which can be removed by 95% TFA without significant cleavage of the peptide aldehyde from the resin (Figure 2). The free tetrapeptide aldehyde was obtained in 92% purity. The scavenging process is slow at ambient temperature and

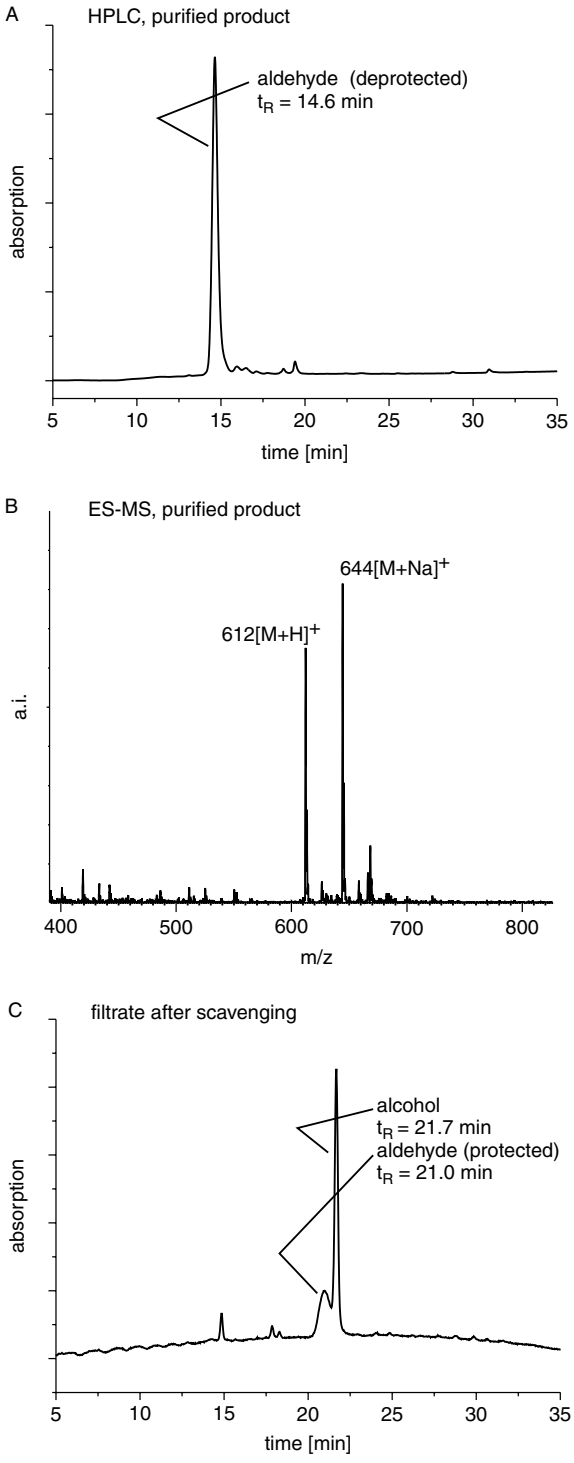


Figure 2 A: HPLC-chromatogram (Purity 92%, $\lambda = 214$ nm; $t_R = 14.6$ min) B: ES-MS spectrum of purified Ac-WEHD-H (**42b**) ($[M + H]^+$: $m/z = 612$, $[M + Na]^+$: $m/z = 644$. C: HPLC-chromatogram of the filtrate; scavenged 3 h at r.t. About 30% aldehyde (**42**) remains in the filtrate ($t_R = 21.0$ min). The alcohol (**24**) eluates at $t_R = 21.7$ min (purity = 63%, $\lambda = 214$ nm).

30% of the aldehyde was still detectable in the filtrate after 3 h. The process had to be repeated to scavenge the aldehyde quantitatively.

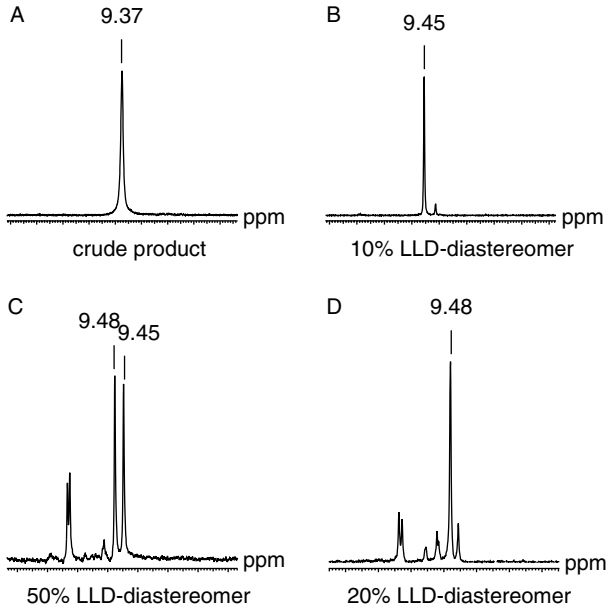


Figure 3 ^{1}H-NMR spectra section of Ac-LLL-H (**37**) A: crude product (**37a**); B: Storage in solution at r.t. for 2 days. (**37c**); C: capture-release procedure at 60 °C (**37d**); D: capture-release procedure at r.t. (**37b**).

Table 4 Chemical Shifts (^{1}H-NMR) of Aldehyde Protons

Peptide aldehyde	δ [ppm] –CHO	Peptide aldehyde	δ [ppm] –CHO
Ac-LLL-H (**37b**)	9.48[b]	Z-LLI-H (**46b**)	9.58[f]
Ac-FVAV-H (**38b**)	9.52[b]	Ac-FVAL-H (**47b**)	9.41[e]
Z-LLV-H (**39b**)	9.55[b]	Ac-FVAI-H (**48b**)	9.57[e]
Ac-FVAG-H (**40b**)	9.16[c]	Ac-FVAS-H (**49b**)	9.19[c]
Ac-FIW-H (**41b**)	9.81[d]	Ac-FVAD-H (**50b**)	9.75[e]
Ac-WEHD-H (**42b**)	—	Ac-A-Thz-D-H (**51b**)	9.58[e]
Ac-YVAD-H (**43b**)	9.41[e]	Ac-FVAK-H (**52b**)	9.18[c]
Z-GFG-H (**44b**)	9.39[c]	Ac-LVAM-H (**53b**)	9.44[e]
Z-IEAL-H[a] (**45b**)	—	Ac-LLM-H (**54b**)	9.15[c]

[a] Glu (E) is *tert.*-butyl protected (**45b**)
[b] $CDCl_3$
[c] d_6-DMSO
[d] d_7-DMF
[e] MeOD
[f] d_6-Acetone

Analysis of Epimers

Earlier publications [21,25] have reported a high degree of epimerization when applying their reaction conditions for peptide aldehyde synthesis. NMR spectroscopy is the best method to quantify epimerization, since aldehyde protons of the diastereomers usually show different chemical shifts. Ac-LLL-H **37b** was selected as an example for a typical ^{1}H-NMR measurement of epimerization rate. The crude tripeptidylaldehyde obtained after IBX-resin oxidation showed no LLD-diastereomer of Ac-LLL-H **37b** (Figure 3A). Within 2 days in the dark at ambient temperature in DCM 10% of the compound was epimerized (Figure 3B). A temperature of 60 °C for the capture-release steps as described in the literature [20] resulted in about 50% epimerization of the peptide aldehyde (Figure 3C). Performing the immobilization at ambient temperature decreased the epimerization to 20% (Figure 3D).

To complete the structural analysis ^{1}H, ^{13}C, ^{1}H^{1}H-COSY and HSQC (^{13}C^{1}H-COSY) NMR-experiments were carried out. The ^{1}H chemical shifts of the aldehyde protons are specified in Table 4. The fully assigned ^{1}H- and ^{13}C-NMR data of Z-LLV-H (**39b**) and of the protected crude Ac-Ala-Thz-Asp(tBu)-H are given as examples.

Determination of Inhibition Constants

Selected peptide aldehydes were tested for their inhibitory effect against caspases 1 and 3. Ac-YVAD-AMC served as a caspase 1 substrate and Ac-DEVD-AMC as a caspase 3 substrate. Caspase activity was determined by measuring the release of AMC (7-amino-4-methyl-coumarin). The commercially available synthetic peptide aldehyde Ac-DEVD-H was used to validate the assay with respect to the determination of inhibitor constants for recombinant caspase 3. The synthetic Ac-YVAD-H (**43b**) was used to validate the caspase assay with recombinant caspase 1. The K_M value for Ac-YVAD-AMC was from the provider, the Michaelis-Menten constant of Ac-DEVD-AMC was found to be 2.8 ($\pm$0.7) $\times$ 10^{-5} M, which is very close to the value 2.4×10^{-5} M presented by Zhou *et al.* [41].

Two new structures from the pool of synthetic peptide aldehydes were chosen for caspase inhibition and tested against caspases 1 and 3. Ac-FVAD-H (**50b**) showed an inhibition constant of 5.2×10^{-9} M against caspase 1 and 3.1×10^{-5} M against caspase 3. Comparing the K_I values of Ac-FVAD-H and Ac-YVAD-H (**43b**) 1.9×10^{-8} M for caspase 1, shows that Ac-FVAD-H has increased inhibitory potency (Table 5). Obviously, the exchange of the *N*-terminal tyrosine by phenylalanine leads to better inhibition by the tetrapeptide aldehyde but still keeps the specificity for caspase 1 as demonstrated by the low K_I value of Ac-FVAD-H (**50b**) against caspase 3.

The novel backbone modified tetrapeptidomimetic aldehyde Ac-A-Thz-D-H (**51b**) was chosen as an example involving structural diversity — it has a 4-aminomethyl-thiazole-2-carboxylic acid (Thz) [38] moiety near the aspartic aldehyde. For this compound specificity between the two caspases could not be detected and no significant inhibition of either enzyme was found: for caspase 1 K_I was 3.0×10^{-5} M, for caspase 3 the constant was determined to be 3.9×10^{-5} M.

The estimated inhibition constants, determined by measuring the steady state rate of substrate

hydrolysis in and without the presence of inhibitor, are summarized in Table 5.

Summary

This report introduces a novel procedure for the solid-phase synthesis and smooth and efficient oxidation of peptide alcohols by the IBX polymer reagent. The method is compatible with the common protecting groups in SPPS as shown by the synthesis of the polyfunctional tetrapeptide aldehyde Ac-WEHD-H (**42b**) containing four protecting groups. The oxidation to the peptide aldehyde proceeds without epimerization and a capture-release protocol using polymer-bound threonine has been optimized to yield only about 20% of the LLD-diastereomer compared with the 50% obtained by previously described procedures.

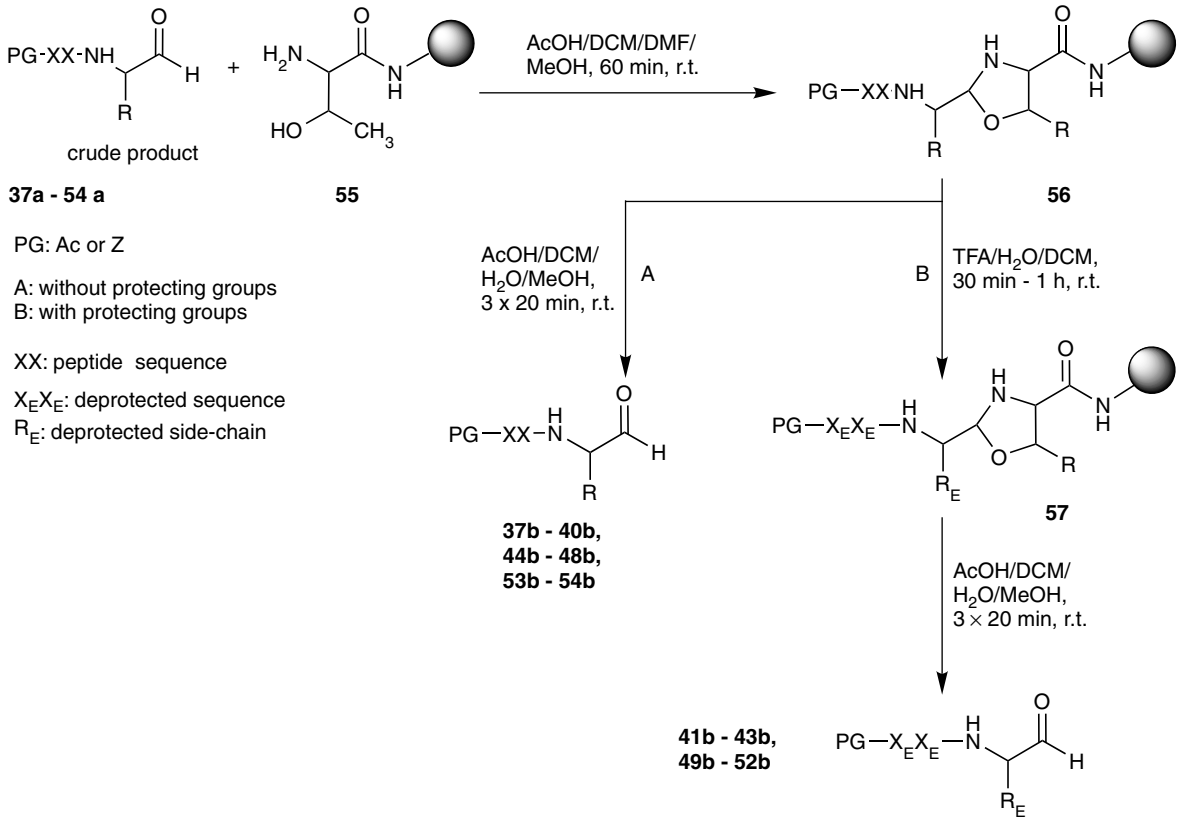
MATERIALS AND METHODS

Chemicals

Solvents, Cs_2CO_3, N-methylmorpholine (NMM), oxone tetrabutylammonium salt and triethylamine were purchased from Fluka (Neu-Ulm, Germany), *tert.*-butyl alcohol from Merck (Darmstadt, Germany). Fmoc-protected amino acids and Merrifield resin were from Merck Biosciences (formerly: Novabiochem, Läufelfingen, Switzerland) and Aldrich (Steinheim, Germany). TCP-resin and threonyl-resin were from PepChem (Tübingen, Germany).

Table 5 Inhibition Constants of Peptide Aldehydes for Caspase-1 and Caspase-3

	K_I caspase-1 [M]	K_I caspase-3 [M]
Ac-DEVD-H (commercial control)		$2.2\ (\pm1.1) \times 10^{-9}$
Ac-YVAD-H (**43b**)	$1.9\ (\pm0.3) \times 10^{-8}$	$7.5\ (\pm0.6) \times 10^{-5}$
Ac-FVAD-H (**50b**)	$5.2\ (\pm0.4) \times 10^{-9}$	$3.1\ (\pm1.3) \times 10^{-5}$
Ac-A-Thz-D-H (**51b**)	$3.0\ (\pm0.2) \times 10^{-5}$	$3.9\ (\pm1.4) \times 10^{-5}$



Scheme 4 Scavenging of peptide aldehydes with threonyl- resin A: Immediate cleavage of the peptide aldehydes after filtration and washing using acetic acid. B: Peptide aldehyde containing Boc-, *tert.*-butyl- and Trt-side-chain protecting groups were deprotected at r.t. Then release of the deprotected peptide aldehyde was carried out with acetic acid.

Chromatography and Spectroscopy

For HPLC analyses a Waters 600S System equipped with an autosampler unit (WISP 712) and UV detection at 214 nm was used. The HPLC column was Nucleosil 100 C18, 250 × 2 mm, 5 μm particle size (Grom, Herrenberg, Germany). LC-MS experiments were performed on a Quattro II spectrometer (Micromass, Altrinchan, UK) coupled to an INTEGRAL™ HPLC system (Perseptive Biosystems, Weiterstadt, Germany) or a Bruker Esquire 3000 plus system (Bruker Daltonics, Bremen, Germany) coupled to an Agilent 1100 HPLC system (Agilent, Waldbronn, Germany). High resolution ESI-FTICR-MS spectra were taken on a Daltonic APEX II spectrometer (Bruker, Bremen, Germany). 2D-NMR spectra were acquired on an AMX 400 MHz spectrometer (Bruker Analytik, Bremen, Germany). The samples were measured in $CDCl_3$ or d_6-DMSO. Thin layer chromatography was performed on fluorescent silica gel plates (60 F254, 5 × 10 cm, Merck, Darmstadt, Germany).

Preparation of IBX-Resins (General Procedure)

The preparation was performed according to the original protocol for small amounts of resin [31], which was scaled up to 10 g of resin.

Attachment of 5-Hydroxy-2-iodomethyl benzoate. Merrifield resin (10 g; initial loading 1.2 mmol/g; 12.0 mmol) and cesium carbonate (2 eq.; 24.0 mmol, 7.8 g) were suspended in DMF (45 ml) and 5-hydroxy-2-iodomethyl benzoate (3 eq.; 36.0 mmol; 10 g) was added. After stirring at 80 °C for 3 h the suspension was allowed to cool to 30 °C. Acetic acid (50 ml) was added and stirring was continued. After 30 min the resin was washed thoroughly with THF, MeOH, DCM, MeOH and Et_2O to give a fine granular resin. IR: 1111 cm^{-1}, 1213 cm^{-1}, 1244 cm^{-1}, 1288 cm^{-1}, 1378 cm^{-1}, 1561 cm^{-1}, 1588 cm^{-1}, 1733 cm^{-1}. Loading: 0.92 mmol g^{-1}. Iodine content: 11.7%.

Ester hydrolysis. The resin was suspended in 1 M $KOSiMe_3$ in dry THF (150 ml), shaken at r.t. for 18 h, washed thoroughly with MeOH and THF. A mixture of THF/AcOH (200 ml; 9 : 1, v : v) was added and shaken for an additional 5 h at r.t. Finally the resin was washed thoroughly with THF, DCM, MeOH and Et_2O.

Formation of IBX polymer reagent. The dry resin was treated with a solution of Oxone™ tetrabutylammonium salt (7 eq.; 77 mmol; 77.7 g; active oxygen ~1.6%) in dry DCM (120 ml). Methane sulfonic acid (7 eq.; 77 mmol; 4.98 ml) was added slowly and agitated at r.t. for 3 h. The resin was washed thoroughly with DCM, Et_2O, DCM, Et_2O, DCM and Et_2O to give a fine granular resin.

The final loading of the IBX-resin was determined by elemental analysis of iodine and ranged between 76% and >95% with respect to the initial loading of the Merrifield resin with chloride (as determined by elemental analysis of chloride). The oxidation of piperonyl alcohol (1 eq. according to the theoretical maximum) was performed to measure the oxidative capacity of the resin [31].

Preparation of Amino Alcohols (General Procedure)

A solution of the Fmoc-α-amino acid (1 eq.; 25 mmol) in dry dimethoxyethane (DME) (50 ml) was cooled to −15 °C. In a nitrogen atmosphere *N*-methylmorpholine (NMM) (1 eq.; 25 mmol; 2.78 ml) and isobutyl chloroformate (ICF) (1 eq.; 25 mmol; 3.4 ml) were added simultaneously and dropwise under stirring at −15 °C. After 10 min, stirring was continued and the reaction mixture was allowed to rise to room temperature within 30 to 60 min.

The mixture was filtered under nitrogen atmosphere to remove precipitated NMM hydrochloride. The filtrate was cooled to −15 °C and a solution of $NaBH_4$ (1.5 eq.; 37.5 mmol; 1.42 g) in degassed water (50 ml) was added. After stirring for 1 min water was added (625 ml). The precipitated Fmoc-amino alcohol was washed with water (100 ml) and *n*-hexane (50 ml).

In the case of oily products, DME was removed under reduced pressure. The water layer was extracted with DCM (100 ml, 3×) and the combined organic layers were washed with water and saturated NaCl solution and dried with Na_2SO_4. Finally the solvent was removed under reduced pressure. Precipitation of the Fmoc-β-amino alcohol occurred after covering the oil with *n*-hexane. Purification was carried out by column chromatography on silica gel (particle size: 0.04–0.062 mm; EtOAc : DCM 1 : 1; NEt_3 0.3%). The eluate was washed with citric acid (30% solution, 50 ml, 3×), dried over Na_2SO_4 and the solvents were removed under reduced pressure. The Fmoc-β-amino alcohols were analysed with TLC and/or HPLC, ES-MS and NMR. In the following the ^{13}C-NMR data of the used Fmoc-L-amino alcohols are given.

Fmoc-Cys(Trt)-ol (1): ^{13}C-NMR (100.62 MHz, $CDCl_3$): δ = 156.6, 144.9, 144.2, 141.7, 130.0, 128.4, 128.1, 127.3, 127.4, 125.4, 120.4, 67.5, 67.1, 64.5, 52.5, 47.6, 33.4.

Fmoc-Asp(OtBu)-ol (2): ^{13}C-NMR (100.62 MHz, d_6-DMSO): δ = 171.1, 156.3, 143.8, 141.3, 127.7, 127.0, 125.0, 120.0, 81.5, 66.8, 64.5, 50.0, 47.2, 37.3, 28.0.

Fmoc-Glu(OtBu)-ol (3): ^{13}C-NMR (100.62 MHz, d_6-DMSO): δ = 172.5, 156.3, 143.8, 141.3, 127.7, 127.0, 125.0, 120.0, 81.5, 64.8, 64.5, 52.0, 47.2, 31.7, 28.0, 26.2.

Fmoc-Phe-ol (4): ^{13}C-NMR (100.62 MHz, d_6-DMSO): δ = 156.0, 144.2, 144.1, 139.7, 129.5, 128.4, 127.9, 127.4, 126.2, 125.5, 120.4, 65.6, 63.4, 54.9, 47.1, 37.0.

Fmoc-Gly-ol (5): ^{13}C-NMR (100.62 MHz, d_6-DMSO): δ = 154.8, 142.6, 139.4, 126.2, 125.7, 123.8, 118.7, 63.9, 58.5, 45.4, 41.7.

Fmoc-His(Trt)-ol (6): ^{13}C-NMR (100.62 MHz, d_6-DMSO): δ = 156.2, 143.0, 141.8, 140.1, 136.0, 136.1, 129.9, 128.9, 128.7, 128.1, 128.0, 124.7, 120.6, 119.5, 75.2, 70.1, 63.4, 54.9, 46.9, 33.8.

Fmoc-Ile-ol (7): ^{13}C-NMR (100.62 MHz, $CDCl_3$): δ = 156.3, 143.2, 140.6, 126.9, 126.3, 124.3, 119.2, 65.8, 62.8, 56.5, 46.6, 35.1, 24.7, 14.8, 10.6.

Fmoc-Lys(Boc)-ol (8): ^{13}C-NMR (100.62 MHz, $CDCl_3$): δ = 156.1, 155.8, 143.3, 140.7, 127.1, 126.5, 124.5, 119.4, 78.7, 66.0, 64.1, 52.3, 46.7, 39.0, 29.8, 29.4, 27.8, 22.0.

Fmoc-Leu-ol (9): ^{13}C-NMR (100.62 MHz, $CDCl_3$): δ = 157.2, 144.3, 141.8, 128.1, 127.5, 125.4, 120.4, 66.9, 66.4, 51.8, 47.7, 40.8, 25.2, 23.4, 22.5.

Fmoc-Met-ol (10): ^{13}C-NMR (100.62 MHz, d_6-*DMSO*): δ = 156.3, 144.2, 141.1, 127.9, 127.3, 125.6, 120.4, 65.5, 63.6, 52.4, 47.2, 31.0, 30.4, 15.0.

Fmoc-Asn(Trt)-ol (11): ^{13}C-NMR (100.62 MHz, $CDCl_3$): δ = 156.9, 144.6, 144.2, 141.7, 129.0, 128.4, 128.1, 127.6, 127.5, 125.5, 120.4, 71.4, 67.3, 64.7, 50.6, 47.6, 39.7.

Fmoc-Arg(Pbf)-ol (12): ^{13}C-NMR (100.62 MHz, $CDCl_3$): δ = 159.2, 157.3, 156.8, 144.2, 141.6, 138.7, 132.9, 132.6, 128.0, 127.4, 125.5, 125.1, 120.3, 118.0, 86.8, 67.0, 65.0, 53.1, 47.5, 43.5, 41.3, 28.9, 28.9, 26.0, 19.7, 18.4, 12.8.

Fmoc-Ser(tBu)-ol (13): ^{13}C-NMR (100.62 MHz, d_6-DMSO): δ = 155.4, 143.7, 141.1, 127.3, 126.5, 125.5, 120.2, 73.5, 65.3, 61.0, 60.7, 53.6, 28.1.

Fmoc-Val-ol (14): ^{13}C-NMR (100.62 MHz, $CDCl_3$): δ = 155.7, 143.5, 141.0, 127.3, 126.7, 124.6, 119.66, 66.2, 63.3, 58.2, 46.9, 28.8, 19.1, 18.3.

Fmoc-Trp(Boc)-ol (15): ^{13}C-NMR (100.62 MHz, d_6-DMSO): δ = 156.2, 149.4, 144.2, 141.0, 135.1, 130.9, 124.6, 123.7, 122.8, 127.9, 127.3, 125.6, 120.4, 119.7, 118.3, 115.0, 83.7, 65.7, 63.5, 53.3, 47.0, 28.0, 26.5.

Fmoc-Tyr(tBu)-ol (16): ^{13}C-NMR (100.62 MHz, d_6-DMSO): δ = 156.0, 153.4, 144.3, 141.1, 134.6, 129.9, 127.9, 127.3, 125.6, 123.7, 120.4, 77.9, 65.7, 63.4, 54.8, 47.1, 36.5, 28.9.

Preparation of Peptide Alcohols

The synthesis of the peptide alcohols was determined to yield 120 μmol product.

Loading of TCP-resin (General Procedure)

Dry TCP-resin (capacity 1 mmol/g) was added to a solution of the Fmoc-L-amino alcohol (1 eq.) and pyridine (4 eq.) or DIEA (4 eq.) in dry DCM. After 12 h of stirring the TCP-resin was filtered off. The remaining trityl chloride groups were capped by a solution of DCM, MeOH and DIEA (17 : 2 : 1; v : v : v) for 30 min. The resin was filtered and washed thoroughly with DMF, DCM, MeOH and Et_2O (3×). The loading capacity was determined via UV spectroscopy of the piperidine cleavage product of the Fmoc-protecting group. The loadings ranged from 0.4 to 0.6 mmol g^{-1}.

Fmoc Deprotection (General Procedure)

The polymer-bound Fmoc amino alcohol (100 mg; loading 0.5 mmol g^{-1}) was suspended in piperidine (20% in DMF; 2 ml) and shaken for 20 min at ambient temperature. The resin was washed with DMF. The deprotection procedure was repeated one more time. The resin was washed thoroughly with DMF (10×), DCM (4×) and MeOH (1×) in an alternating order and dried under reduced pressure.

DIC/HOBt Coupling (General Procedure)

A solution of Fmoc-amino acid (0.2 mmol; 4 eq.) and HOBt (40 mg; 0.2 mmol; 4 eq.) in dry DMF (1 ml) was added to the polymer bound Fmoc-deprotected amino alcohol (100 mg, loading: 0.5 mmol g^{-1}) swollen in DMF (1 ml). DIC (33 μl; 0.2 mmol; 4 eq.) was added to the suspension and shaken for 1 h at r.t. The resin was filtered and washed thoroughly with DMF, DCM, MeOH, DCM and Et_2O (5×). The completeness of the coupling step was verified with the Kaiser test [37].

Cleavage of the Peptide Alcohols (General Procedure)

A mixture from DCM, HFIP and AcOH (68 : 30 : 2; v : v : v) was added to the resin bound peptide alcohol and shaken for 24 h at r.t. The resin was filtered off and washed with DCM and MeOH (2×). The combined filtrates were dried under reduced pressure and lyophilized from *tert.*-butyl alcohol/water (4 : 1, v : v). The products were characterized using HPLC, ESI-MS and NMR spectroscopy.

Oxidation of (Side-chain Protected) Peptide Alcohols (General Procedure)

A solution of the peptide alcohol (100 μmol) in DCM (300 μl) was added to IBX-resin (2 eq.). Depending on the peptide alcohol it was necessary to add DMF or DMSO to achieve a clear solution. The suspension was stirred for 2 h at r.t. Finally, the resin was filtered off and washed thoroughly with DCM and MeOH (2×). The combined filtrates were dried under reduced pressure and lyophilized from *tert.*-butyl alcohol/water (4 : 1, v : v). The products were analysed using HPLC, ESI-MS and NMR spectroscopy.

Ac-Ala-Thz-Asp(tBu)-H (51a):. **^{1}H-NMR**, **^{1}H-COSY** (400, 16 MHz; $CDCl_3$): δ = 9.63 (s, 1 H), 8.25 (t, ^3J = 8.72 Hz, 1 H), 7.62 (s, 1H), 6.43 (dd, ^2J = 16.17 Hz, ^3J = 7.33 Hz), 4.75 (dd, ^3J = 7.83 Hz, ^3J = 2.78 Hz, 1 H), 4.63 (d, ^3J = 5.05 Hz, 2 H), 4.52 (dd, ^3J = 11.87 Hz, ^3J = 6.82 Hz, 1 H), 2.88 (m, 1 H), 1.93 (s, 3 H), 1.38 (d, 6 H), 1.35 (d, ^3J = 6.82 Hz, 1 H), 1.20 (s, 9 H).

^{13}C-NMR (100.62 MHz, $CDCl_3$): δ = 197.58, 171.82, 169.29, 166.95, 160.06, 147.70, 123.61, 81.21, 68.23, 53.85, 47.68, 39.96, 34.33, 30.20, 27.02, 22.08, 16.68.

Purification via Capture-Release

Capturing by scavenger resin (general procedure). A solution of crude peptide aldehyde (100 μmol) dissolved in a mixture from MeOH/DCM/DMF (30 : 3 : 2; v : v : v + 1% AcOH) (2 ml) was added to threonyl-scavenger resin (100 mg, 100 μmol; 2.05 eq.) and stirred for 1 h at r.t. The resin was washed with DCM (3×) and dried under reduced pressure.

Cleavage of Boc- and tert.-butyl protecting groups (general procedure). Peptide aldehyde attached to threonyl-scavenger resin (100 mg) was treated with a mixture of TFA, H_2O and DCM (90 : 5 : 5; v : v : v) (2.5 ml) for 0.5 h to 1 h at r.t. The resin washed with DCM (3×).

Cleavage of Trt-protecting groups (general procedure). Peptide aldehyde attached to threonyl-scavenger resin (100 mg) was treated with a mixture from TFA, H_2O and TIS (95 : 2.5 : 2.5; v : v : v) (2.5 ml) for 0.5 h to 1 h at r.t. The resin was washed with DCM (3×).

Release from scavenger resin (general procedure). Peptide aldehyde attached to threonyl-scavenger resin (100 mg) was

treated with a mixture from AcOH, H_2O, DCM and MeOH (10 : 5 : 5 : 80; v : v : v : v) (3×, 2 ml) for 20 min at r.t. The resin was filtered off and washed with DCM (2×). The combined filtrates were dried under reduced pressure and lyophilized from *tert.*-butyl alcohol/water (4 : 1, v : v). The products were characterized using HPLC, ES-MS and NMR-spectroscopy.

NMR-data for Z-LLV-H (39b):. ^{1}**H-NMR** (400 MHz, $CDCl_3$) δ = 9.55 (s, 1H), 7.28–7.19 (m, 5H), 6.69 (d, 1H, J = 6.82 Hz), 6.40 (s, 1H), 5.14 (d, 1H, J = 6.06 Hz), 5.03 (d, 2H, J = 4.29 Hz), 4.43 (m, 1H), 4.41 (m, 1H), 4.18 (s, 1H), 2.29 (m, 1H), 1.65 (m, 2H), 1.65 (m, 2H), 1.52 (m, 2H), 0.96–0.83 (m, 6H), 0.96–0.83 (m, 12H).

^{13}**C-NMR** (100 MHz, $CDCl_3$) δ = 199.9, 172.8, 172.4, 156.7, 136.4, 129.6, 129.3, 129.1, 67.6, 63.6, 54.7, 53.0, 41.4, 41.1, 29.3, 25.1, 25.2, 23.2, 22.4, 19.4, 18.2.

Caspase Assays

Enzymes and substrates. The enzyme assays used purified recombinant human caspase-1 from Calbiochem (Schwalbach, Germany) and caspase-3 from R&D Systems (Abingdon, UK) at a reported purity >95%. Caspase-1 substrate Ac-YVAD-AMC was from Calbiochem and as a caspase-3 substrate Ac-DEVD-AMC (from Bachem, Heidelberg, Germany) was used. Ac-DEVD-H (Bachem) was used as an inhibition control.

Enzyme assays. The activity of caspase-3 and caspase-1 was determined by measuring the release of AMC (7-amino-4-methyl-coumarin). Excitation wavelength was set to 380 nm and emission wavelength to 440 nm. All measurements were carried out on a Perkin-Elmer LS50B luminescence spectrometer coupled with the FL WinLab software (Perkin-Elmer, Rodgau-Jügesheim, Germany) at 37 °C. The value of K_M for caspase-1 was taken from the supplier (K_M = 8 μM), whereas the K_M value for the cleavage of Ac-DEVD-AMC by caspase-3 was determined by measuring and plotting the initial rate, v, over a range of substrate concentration S (1 μM up to 100 μM), and fitting the data directly to the Michaelis-Menten equation.

Inhibition constants were analysed by the method of progress curves, where a reaction was initiated by the addition of enzyme to a mixture of substrate and inhibitor. Inhibitor was kept at a large excess of enzyme to render the reaction pseudo-first order for convenience of analysis. The 100 μl reaction was started by adding enzyme to a mixture of substrate (10 μM) and various concentrations of inhibitor in buffer (20 mM HEPES, 10 mM DTT, 100 mM NaCl, 10%(v/v) glycerol, 0.1% CHAPS, pH 7.5). The lowest inhibitor concentration was at least 100-fold that of the enzyme. A sample without inhibitor served as a control for all progress curve experiments to ensure that no substrate depletion or spontaneous enzyme inactivation occurred. All samples were preequilibrated at 37 °C for 5 min before mixing. Also the substrate inhibitor mixture was preequilibrated for 5 min before adding the enzyme.

The observed inhibition constant in the presence of substrate, $K_i(app)$, is given by,

$$\frac{v_0}{v_I} = 1 + \frac{I}{K_I(app)}$$

where v_I is the steady state rate of substrate hydrolysis in the presence of inhibitor concentration I, and v_0 is the uninhibited rate [42]. The overall equilibrium constant is obtained by taking into account the substrate concentration S according to the following equation

$$K_I = \frac{K_I(app)}{1 + \frac{S}{K_M}}.$$

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