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Covalent capture purification of polypeptides after SPPS via a linker removable under very mild conditions

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Abstract—The covalent purification of polypeptides possessing an N-terminal cysteine or threonine residue via formation of a thiazolidine or oxazolidine with an aldehyde-functionalized-resin has been successfully demonstrated. To extend the applicability of this approach to any possible N-terminal residue, a special linker derived from (*S*)-4-amino-2-hydroxy-butyric acid was incorporated into peptidyl-resin. This linker represents the connecting point between the capture unit (cysteine) useful for the isolation of the desired polypeptide and the desired N-terminus. The target polypeptide was recovered by periodate oxidation, which cleaved the covalent bond between the linker and the last residue of polypeptide under very mild conditions.

After solid-phase peptide synthesis (SPPS), whether the Boc or Fmoc strategy is used, the purification step is a major problem to obtain the desired peptide with a satisfactory yield, completely devoid of the accumulation of truncated or deleted chains generated on the resin.

Many groups[1–6] have sought to functionalize the N-terminal residue of full-length peptides with a removable purification handle as a final step in SPPS. After cleavage from the resin, these handled-peptides can be specifically captured from a complex mixture by chemoselective reaction or affinity chromatography. A variety of handles have already been reported in the literature, including ones used in covalent[1] or in affinity-type purification of the desired peptide.[2–8] However, none of these methods can be acceptable for a fully deprotected peptide due to the harsh conditions required for handle removal (cyanogen bromide, piperidine, high pH). Therefore, their use has been limited.

As reported recently in the literature,[9] covalent purification of polypeptides possessing either N-terminal cysteine or threonine residue can be successfully applied after SPPS to isolate the desired compound via the specific formation of a thiazolidine or oxazolidine ring.[10]

Here, we present an extension of this approach using a special linker, the 4-amino-2-hydroxy-butyryl group (Ahb) linked to the last residue of a growing peptide chain. As shown in Fig. 1, we synthesized an appropriate Ahb derivative (**4**) before coupling it into peptidyl-resin, starting from commercially available (*S*)-4-amino-2-hydroxy-butyric acid (**1**). After Boc deprotection, we coupled a cysteine residue to the free amine. This generated a peptide possessing all the characteristics necessary for covalent capture purification.

One great advantage of using the Ahb group in protein purification is demonstrated by the removal conditions: this group can be efficiently and completely removed by periodate oxidation ($NaIO_4$), a procedure known not to

Abbreviations: HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3,tetramethyluronium hexafluorophosphate; BOP, 2-(1*H*-benzotriazole-1-yl)-oxy-tris(dimethylamino)phosphonium hexafluorophosphate; HOAt, 1-hydroxy-7-aza-benzotriazole; DIEA, diisopropylethylamine; DCCI, dicyclohexylcarbodiimide; $NaBH_3CN$, sodium cyanoborhohydride; BrBzl, bromide benzyl; AcOH, acetic acid; Boc_2O, di-tert-butyl dicarbonate; Boc, tert-butyloxycarbonyl; $LiAlH_4$, lithium aluminum hydride; ZCl, benzyloxycarbonylchloride; $HCl{\cdot}HN(OCH_3)CH_3$, *N*,*O*-dimethylhydroxylamine hydrochloride; $NaIO_4$, sodium metaperiodate; TFA, trifluoroacetic acid; HF, liquid hydrogen fluoride; DCM, dichloromethane; DMF, dimethylformamide; THF, tetrahydrofuran; MeOH, methanol; SPy, *S*-pyridine; TCEP, tris(2-carboxyethyl) phosphine hydrochloride; CH_3CN, acetonitrile; HPLC, reversed-phase high performance liquid chromatography; rt, room temperature.

Keywords: covalent capture; purification; removable linker; periodate oxidation; large polypeptide.

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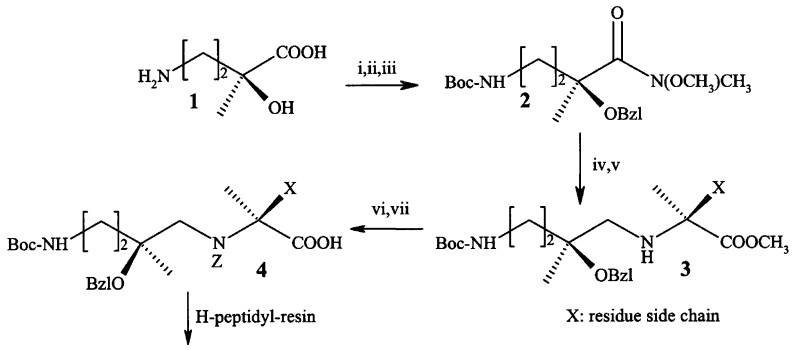

Figure 1. Synthetic route used for the preparation of Ahb derivative. *Reagents and conditions*: (i) Boc_2O, NaOH 1N, rt, H_2O/dioxane, overnight; 94%; (ii) BrBzl, NaH, 0°C, CH_3CN, 4 h; 80%; (iii) $HCl{\cdot}HN(OCH_3)CH_3$, BOP, DIEA, rt, DMF, 3 h; 85%; (iv) $AlLiH_4$, 0°C, THF, 1 h, then HCl·2HN-AA-OMe (where AA corresponds to glycine side chain), $NaBH_3CN$, MeOH/AcOH 1%, rt, 3 days; 41%; (v) ZCl, DIEA, rt, THF, overnight; 95%; (vi) NaOH 2N, THF, 0°C, 2 h, 77%.

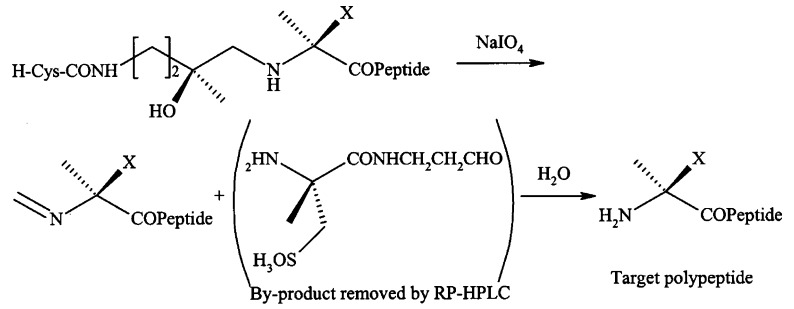

Figure 2. Periodate-assisted-cleavage of our Ahb group. Variable excess of $NaIO_4$ reagent or methionine scavenger are applied depending on the type of X (X: residue side chain) or the presence of an internal methionine in the sequence. Buffer: imidazole 50 mM, methionine 50–150 equiv. over the Ahb-peptide., $NaIO_4$ 5–30 equiv., pH 6.95. Final concentration of peptide 0.5 mM. Reaction time 5 min followed by quenching the reaction with ethylene glycol and acetic acid (5% of the reaction solution), rt.

damage proteins when carefully controlled[11] (see Fig. 2). Indeed, this oxidizing agent is able to reverse a reductive alkylation since it can recognize and cleave a 1-amino-2-ol bond like that found in our Ahb group.[12,13]

The feasibility of using the 4-amino-2-hydroxy-butyryl group in polypeptide purification was tested with two model peptides of sequence MYAKYAKL (**5**) and GGCAVVFVTRKNRQVSANPEKKAVREYINSLELA (**6**) (Table 1). These two latter were assembled on the resin using Boc chemistry with optimized HBTU/DIEA in situ neutralization coupling cycles.[14]

Following deprotection of the N-terminal residue of these peptides, the protected Ahb group was manually introduced via DCCI/HOAt activation. After deprotection of the amino group of the Ahb group, attachment of Boc-Cys(pMeBzl)-OH (capture unit) and HF cleavage, crude final peptides (**7**) and (**9**) were mixed with an aldehyde-functionalized-resin for 6 h. After washes to eliminate the incomplete sequences, the release step was performed in presence of *O*-methylhydroxylamine hydrochloride at acidic pH (see Figs. 3 and 4). The covalent capture procedure in this case was able to remove all the side products not possessing N-terminal cysteine. However, we observed some impurities eluting after peptides (**7**) and (**9**) (see Figs. 3 and 4), corresponding to post-HF cleavage modifications (according to mass spectrometry, data not shown).

Prior to periodate oxidation, the internal cysteine of peptide (**9**) was protected with the *S*-pyridyl group followed by HPLC purification to avoid thiol oxidation to a sulfonate derivative. Finally, the standard $NaIO_4$ cleavage procedure (as used during oxidation of serinyl-peptides to glyoxylyl ones)[15] was used for the removal of Cys-Ahb group both from peptides (**7**) and (**10**). The internal cysteine of peptide (**11**) was then liberated with the TCEP reagent (see Fig. 4). Because of the length and the presence of critical residues like cysteine, tryptophane (peptide **6**) and methionine (very close to the cleavage site performed by $NaIO_4$, peptide **5**) in the test

Table 1. List of synthetic peptides

5	MYAKYAKL
6	GGCAVVFVTRKNRQVSANPEKKAVREYINSLELA
7	C-Ahb-MYAKYAKL
8	Ac-YAKYAKL
9	C-Ahb-GGCAVVFVTRKNRQVSANPEKKAVREYINSLELA
10	C(SPy)-Ahb-GGC(SPy)AVVFVTRKNRQVSANPEKKAVREYINSLELA
11	GGC(SPy)AVVFVTRKNRQVSANPEKKAVREYINSLELA

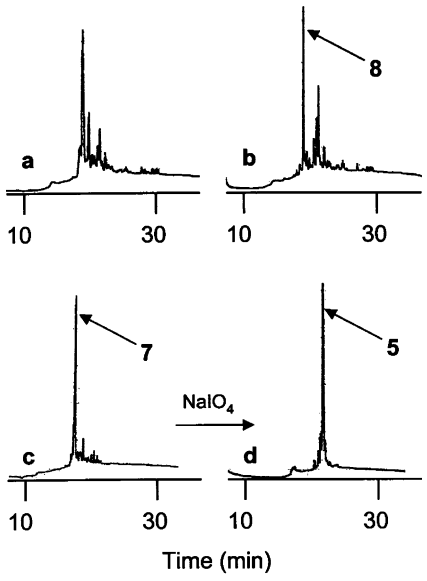

Figure 3. Covalent capture–release concept and linker removal by $NaIO_4$ of peptide **7**. Top, (a) analytical HPLC of crude material, (b) of the supernatant after 6 h of reaction time. After washes, (c) the liberated release of the peptide from the resin in the presence of 200 mM *O*-methylhydroxylamine hydrochloride solution (H_2O/CH_3CN 1:1) is performed. Bottom, (d) periodate cleavage of peptide **7**'s Cys-Ahb group with 5 equiv. of $NaIO_4$ for 5 min and analytical HPLC of crude material. Capture and washing buffer: MES 100 mM, TCEP 10 mM, pH 6.4, H_2O/CH_3CN 1/1. Volumes injected on HPLC were kept constant.

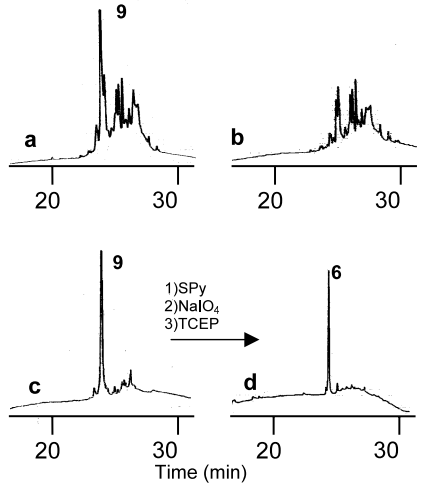

Figure 4. Covalent capture–release concept and linker removal of peptide **9**. (a) Analytical HPLC of crude material, (b) of the supernatant after 6 h of reaction time and (c) liberated peptide from the resin after the washing step (buffer, see Fig. 3). Volumes injected were kept constant. (d) Product of periodate cleavage of peptide **9**'s Ahb group after deprotection of internal cysteines.

peptides, we can affirm that periodate-assisted-removal of the Cys-Ahb group represents a great advantage for the recovery of the target polypeptides devoid of any side reaction on these sensitive sequences.

Moreover, we checked the efficiency of periodate cleavage on other N-terminal amino acids using a series of three Ahb-dipeptides, (**12**), (**13**) and (**14**) containing either bulky residues such as Leu, Ile or a charged side chain (aspartic acid, see Table 2). No major problems were encountered with our compounds, when the standard conditions for this reaction were employed. As shown in Table 2, the Ahb group has been successfully removed in 5 min according to RP-HPLC and MS (data not shown), confirming the great lability of α-amino alcohols and the possibility to reverse the reductive alkylation using $NaIO_4$ as an oxidizing agent.[12,13]

In conclusion, we propose a new approach which facilitates the synthesis and purification of large polypeptides. It consists of two key features. In a first step, polypeptides possessing an amino-terminal Cys or Thr residue are purified by forming a covalent bond (thiazolidine or oxazolidine) as reported in the literature.[9] Alternatively, polypeptides which do not have an amino-terminal Cys or Thr residue are derivatized with an auxiliary capture tag (Cys-Ahb group), containing both the capture unit (cysteine) and a removable linker (characterized by a vicinal 1-amino-2-hydroxy group). After the covalent capture-release cycle,[9] the Cys-Ahb group is efficiently and quantitatively removed under gentle conditions through oxidation with $NaIO_4$ within 5 min (see Fig. 2 and Table 2).

Finally, the peptide chemist has more efficient tools to produce target proteins of pharmacological interest with a high purity, by combining (i) improved stepwise solid-phase peptide synthesis; (ii) an acetylation step after each coupling reaction; (iii) a covalent purification method devoid of potential side reactions on proteins as reported in this article and (iv) using the well-known Native Chemical Ligation.[15] Special procedures are under development to apply covalent capture purification to N-terminal prolyl, pyroglutamyl, acetyl or other N-blocked polypeptides.

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Table 2. Periodate cleavage of α-amino-alcohol moiety with more hindered environment. For experimental conditions, see Fig. 2

Ahb-peptides	Excess of $NaIO_4$ reagent used for Ahb group removal from Ahb-peptides (equiv.)
Ahb-Leu-Phe-OH (**12**)	30
Ahb-Ile-Phe-OH (**13**)	30
Ahb-Asp-Phe-OH (**14**)	5

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