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Synthesis of a thioester linker precursor for a general preparation of peptide C-terminal thioacids

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Abstract—A general procedure to prepare peptide thioacids by solid-phase peptide synthesis is presented. The method involves the synthesis of 4-[α-(*S*-acetyl)mercaptobenzyl]phenoxyacetic acid as general precursor. This reagent once attached to a solid support is derivatized with the Boc-amino acid of choice after deprotection of the thiol.

Peptide α-thioacids are key intermediates in the synthesis of proteins by chemical ligation of unprotected peptides.[1–3] The key feature of the thioacid moiety lies in its ability to act as a nucleophile at pH 3–6, in a unique window where all nucleophiles present in peptides and proteins are basically unreactive. Thus, a peptide-$^{\alpha}$COSH can be easily converted into a peptide thioester by reaction in aqueous acidic conditions with either an alkyl bromide or with an activated symmetrical disulfide. A case in point is the coupling of a peptide-$^{\alpha}$COSH with the alkyl bromide at the N-terminus of another segment generating a single polypeptide chain with an artificial thioester bond at the ligation site.[2] Furthermore due to its unique reactivity this functionality has also been exploited in (i) the convergent chemical ligation of multiple unprotected peptide segments (when the protein multiple sub-domains are synthesized in parallel by ligation of two fragments with a normal thioester strategy, and the final protein is generated by ligation of the sub-domains after activation of the thioacid)[3] and more recently (ii) in extended chemical ligation involving a removable auxiliary group[4] when a highly reactive thioester is required.[5]

Thioacid peptides are generally synthesized on the appropriate Boc-aminoacyl-*S*-resin as described by Kent.[6] This procedure however has some disadvantages[6] since it requires the preparation in solution of the Boc-aminoacyl-*S*-linker-COOH for each amino acid before attachment to the amino-methyl resin. Therefore the development of a unique resin precursor that allows the loading of the amino acid or the reagent of choice is crucial to offer a more attractive route to thioacid peptides in a straightforward manner.

In this paper we report the synthesis of 4-[α-(*S*-acetyl)mercaptobenzyl]phenoxyacetic acid in a four-step route. This reagent is used to generate a general resin for thioacid peptide synthesis, which can be functionalized with the Boc-amino acid of choice. The preparation of this precursor is described in Scheme 1.

The 4-(α-hydroxybenzyl)phenoxyacetic acid was easily synthesized in three steps from the corresponding 4-hydroxybenzophenone using the reported synthetic pathway.[7] The problems outlined by Goldstein[8] could be overcome with a supplementary purification step of the 4-benzoylphenoxyacetic acid ethyl ester by ethyl acetate extraction, washing and crystallization before the saponification step. The 4-(α-hydroxybenzyl)phenoxyacetic acid was isolated as the DCHA salt in a 55% yield. The corresponding free acid was then reacted with concentrated HCl and the resulting 4-(α-chlorobenzyl)phenoxyacetic acid crystallized upon addition of petroleum ether and was isolated in a 79% yield.[9] Reaction of CH_3COSH in a 1.1 M excess over the chloro-substituted linker in the presence of equimolar amounts of DIEA in DMF was almost quantitative after 20 min incubation at room temperature, generating

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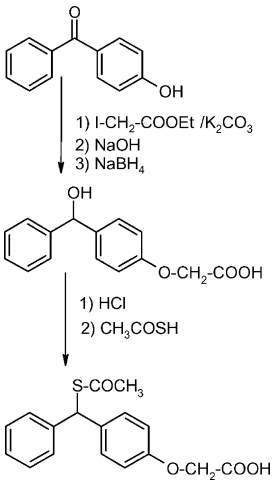
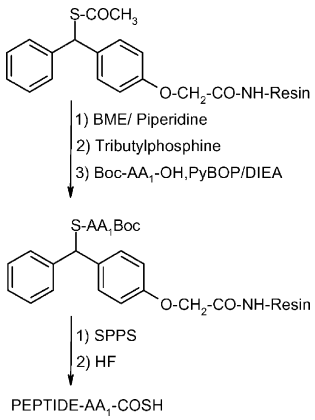


Scheme 1. General synthesis of 4-[α-(*S*-acetyl)mercaptobenzyl]phenoxyacetic acid.

after acidification, extraction with ethyl acetate and washing with 0.5 M H_2SO_4, the desired thioester 4-[α-(*S*-acetyl)mercaptobenzyl]phenoxyacetic acid as an oil in 79% yield.[10] As shown in Scheme 2, the linker, once coupled to an amino-methyl resin and deprotected, can react with any of a range of Boc-amino acids, using standard conditions. Then our deprotection protocol employing β-mercaptoethanol (BME) and piperidine in DMF[11] directly on the resin allows the convenient and facile loading of the first amino acid. The resin is then further elongated to produce the desired peptide thioacid after acid cleavage with HF. Incubation of the deprotected thiol intermediate with tributylphosphine before amino acid functionalization allows the elimination of the β-mercaptoethanol adduct, thus increasing significantly the substitution level of the resin.

Using this deprotection protocol employing BME and piperidine in DMF,[11] loading of the resin was evaluated for different Boc-amino acids. As shown in Table 1, comparable loadings were obtained with the three amino acids investigated.

Boc-Gly loaded resin was used in the synthesis of the peptide Tyr-Ala-Lys-Tyr-Ala-Lys-Leu-Tyr-Arg-Ala-Gly-SH by solid phase. The crude peptide obtained after HF cleavage from the resin was analyzed by RP-HPLC as shown in Figure 1. The major component presented the expected mass value (M+H, m/z calcd 1320.6; found 1320.4). The yield of the correct product was consistent with the same product obtained using a classical thioacid resin generated with previous technology.[6]



Scheme 2. General route to peptide C-terminal thioacids.

Table 1. Substitution of the thioester resin loaded with different amino acids

Boc-amino acid	Substitution (mmol/g)[a]
Boc-Gly	0.33
Boc-Ala	0.33
Boc-Arg	0.30

[a] Evaluated by quantitative ninhydrin test. The substitution of the starting resin is 1 mmol/g.

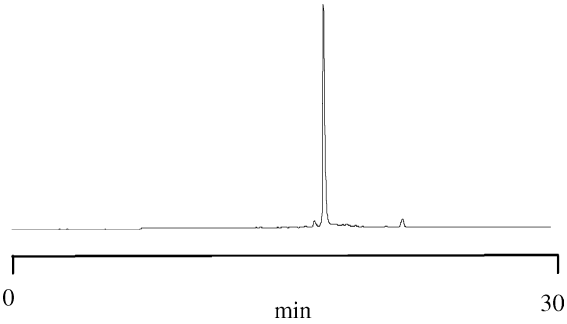


Figure 1. RP-HPLC of the crude model peptide α-thioacid.

In conclusion, we have significantly improved the preparation of peptide-$^{\alpha}$COSH as demonstrated by the synthesis of the peptide Tyr-Ala-Lys-Tyr-Ala-Lys-Leu-Tyr-Arg-Ala-Gly-SH by solid-phase synthesis. The coupling of the 4-[α-(*S*-acetyl)mercaptobenzyl] phenoxyacetic acid proceeds smoothly under standard conditions and the amino acid of choice can be easily incorporated in good yields. Furthermore our ability to functionalize the resin with the reagent of choice will enable the generation of peptide libraries of peptide-$^{\alpha}$COSH.

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9. The two intermediates were characterized by ESI-MS; 4-(α-hydroxybenzyl)phenoxyacetic acid, $C_{15}H_{14}O_4$ $M=$ 258.3 $(M-H)^-$ 257.3; the 4-(α-chlorobenzyl) phenoxyace-

tic acid intermediate could only be detected as the dehalogenated form: 4-(α-chlorobenzyl)phenoxyacetic acid, $C_{15}H_{13}O_3Cl$ $M = 276.7$ $(M-Cl)^+$ 241.2.

10. A typical experimental procedure is as follows: 4-(α-chlorobenzyl)phenoxyacetic acid (9 g, 32.5 mmol) and thioacetic acid (2.55 mL, 35.8 mmol) were dissolved in 35 mL DMF and DIEA (6.2 mL, 35.8 mmol) was added. After being stirred for 20 min at room temperature, the solution was acidified with acetic acid and poured into 300 mL of ethyl acetate, washed with H_2SO_4 0.5 M (3×50 mL) and saturated NaCl (2×50 mL). The ethyl acetate solution was dried over anhydrous Na_2SO_4, filtered and evaporated to give 8 g of 4[α-(*S*-acetyl)mercaptobenzyl] phenoxyacetic acid as an oil in 79% yield. ESI-MS, $C_{17}H_{16}O_4S$ $M = 316.4$ $(M-H)^-$ 315.5.
11. After coupling the 4[α-(*S*-acetyl)mercaptobenzyl]phenoxyacetic acid linker to the amino-methyl resin under standard conditions, the resin was incubated with a solution of 2 M β-mercaptoethanol and 2 M piperidine in DMF (2×20 min), then thoroughly washed with DMF and incubated with a 0.5 M solution of tributylphosphine in NMP/H_2O (9/1, v:v), 2×30 min. The resin was washed with DMF and reacted with the Boc-amino acid (10 equiv) preactivated with PyBOP (9 equiv) and DIEA (20 equiv) in DMF for 2 h. The resin was washed and ready for further SPPS.