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Pyroacm Resin: An Acetamidomethyl Derived Resin for Solid Phase Synthesis of Peptides through Side Chain Anchoring of C-Terminal Cysteine Residues

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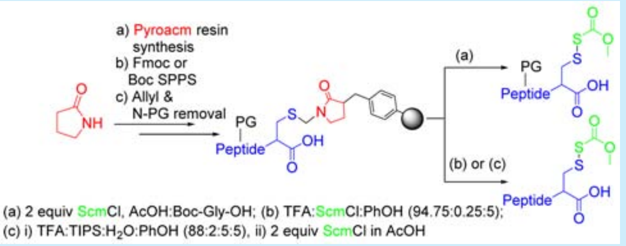
Supporting Information

ABSTRACT: The design, synthesis and utilization of an efficient acetamidomethyl derived resin for the peptide synthesis is presented using established Fmoc and Boc protocols via side chain anchoring. Cleavage of the target peptide from the resin is performed using carboxymethylsulfenyl chloride under mild conditions which gave *in situ* thiol-sulfenyl protection of the cysteine residues. The utility of the resin is successfully demonstrated through applications to the syntheses of model peptides and natural products Riparin 1.1 and Riparin 1.2.



Peptides containing a C-terminal cysteine and cyclic disulfide are highly abundant in nature and are employed as valuable biological probes.[1] Following its development by Merrifield, solid phase peptide synthesis (SPPS) has been employed for industrial and laboratory scale syntheses of numerous synthetic peptides and proteins.[2] Unlike almost all of the other side chain functionality found in the 20 naturally occurring amino acids utilized in SPPS, the free thiol in cysteine is highly reactive and typically requires a trityl (Trt) or acetamidomethyl (Acm, Figure 1, **1a**) protection during peptide chain elongation.[3] Whereas the Trt protective group is acid labile, the Acm group can tolerate both acidic and basic conditions ranging from hydrofluoric acid to hydrazine. Moreover, it is stable under chemical ligation, desulfurization and various reduction conditions including Zn in AcOH.[3b,4] The stability of the Acm group makes it compatible with manipulations used in *tert*-butyloxycarbonyl (Boc)/benzyl (Bn) and fluorenylmethyloxycarbonyl (Fmoc)/*t*-Butyl (tBu) guided peptide assembly and cleavage. Furthermore, the Acm group has been modified to produce even more stable or orthogonal thiol protecting groups. The modifications include trimethylacetamidomethyl group (**1b**),[5a] *S*-phenylacetamidomethyl (**1c**),[5b] Hgm (**1d^2**),[5c] Hqm (**1d^3**),[5c] phthalimidomethyl (**1d^1**),[5d] and trifluoroactamidomethyl (Figure 1, **1e**).[5e]

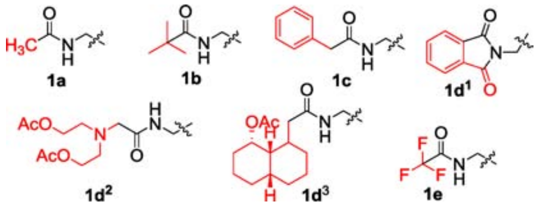


Figure 1. Structures of acetamidomethyl **1a** and its modified protective groups.

Despite these advances, the main challenge associated with protection of C-terminal cysteine residues results from their ready β-elimination and racemization reactions, which result in formation of byproducts during each Fmoc SPPS coupling cycle.[6] To prevent β-elimination, side chain anchoring techniques have been devised. One involves linking a terminal cysteine to a trityl,[7a,d] 2-chlorotrityl,[7b] or Xal[7c] resin through a thioether bond and the other to a masked carboxylic group at the C-terminal.[7e] Unfortunately, the linkers and resins currently employed for side chain immobilization are acid labile resulting in partial cleavage under conditions that induce deprotection of other protected amino acid residues.

To facilitate pre- or postcleavage, side chain modification of other amino acid residues such as those required for installation of a fluorophore or an oligo-saccharide, an analogue of the Acm linker for cysteine thiol protection, is needed, and the resulting process needs to be compatible with both the Fmoc and Boc protocols. Unfortunately, the Acm group and most of its relatives require Hg^{2+}, Ag^{+}, or Tl^{3+} salts for deprotection.[3b,4,8] These toxic heavy metals are hazardous to the environment. Moreover, it is difficult to remove thiol-metal complexes from product mixtures. Carboxymethylsulfenyl chloride (ScmCl) is a reagent that serves as an inexpensive and viable alternative for Acm group removal.[9a] Moreover, the cleavage process leads to *in situ* formation of a sulfenyl group protection at cysteine thiol. Despite its potential advantages, this process has only been explored using fully protected peptides in both solid and solution phase synthesis.[9] As a part of our high-throughput solid-phase synthesis program,[10] we required the availability of

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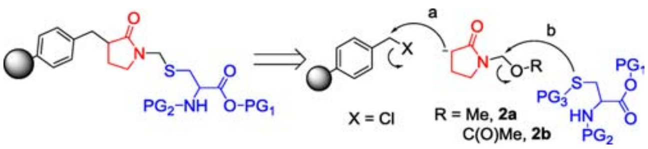
a facile and rapid procedure for preparation of cysteine containing peptide and peptidometic molecules.

Herein, we designed and synthesized a mimic of the Acm linker, termed Pyroacm, which can be employed to anchor terminal cysteines via the side chain thiol moiety. In addition, we developed cleavage strategies for pre- and postmodifications of peptides.

We hypothesized that it should be possible to couple 1-(methoxymethyl)pyrrolidin-2-one (**2a**) or (2-oxopyrrolidin-1-yl)methyl acetate (**2b**) to functionalized resins using C−C bond forming enolate-alkylation reactions. In addition, we believed that coupling of the cysteine residue to the Pyroacm moiety on the resin would take place through thioether bond formation under acidic conditions (Scheme 1).

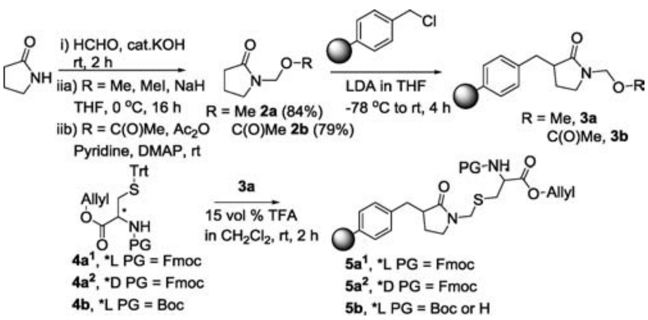
Scheme 1. Preparative Route for Implementation of Pyroacm Based Strategy for Immobilization via a C-Terminal Cysteine Residue[a]



[a]Reagent and conditions: (a) Installment of the Pyroacm linker on standard Merrifield resin using nucleophilic displacement with enolate of **2a** or **2b**. (b) Immobilization of the initial cysteine residue under acidic conditions.

To evaluate this proposal, we first prepared two Pyroacm functionalized linkers **2a** and **2b**. The syntheses began with the treatment of commercially available pyrrolidin-2-one with formalin in the presence of KOH (Scheme 2). Subsequent

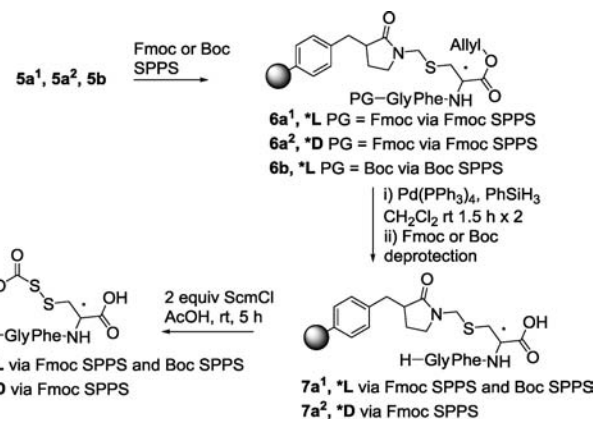
Scheme 2. Synthesis of the Pyroacm Resin and Trans-Thioetherification of Cysteine Derivative onto Pyroacm Resin



methylation of the formed carbinolimide with MeI in THF containing NaH gave the methyl ether derivative **2a** (84%), whereas acetylation of this intermediate produced the ester **2b** (79%). The result of screening various bases and additives showed that installment of **2a** on standard chloromethyl derivatized Merrifield resin (∼2.2 mmol/g) is most effectively accomplished by reaction of the enolate of **2a** generated by treatment of LDA in THF, see Table S1. This process can be utilized to generate the Pyroacm linked resin **3a** on a multigram scale. The ATR-FTIR spectra of **3a** (Figure S5) contains intense and sharp peak at 1691 cm^{-1} corresponding to the pyrrolidone carbonyl group. Introduction of the ester **2b** to the Merrifield resin was unsuccessful owing to its polymerization during enolate formation. Immobilization of an appropriately protected cysteine residue on resin **3a** was explored using Fmoc-L-Cys(Trt)-OAllyl (**4a^1**), Fmoc-D-Cys(Trt)-OAllyl (**4a^2**), and Boc-L-Cys(Trt)-OAllyl (**4b**), prepared by allylation of commercially available substrates (see Supporting Information (SI)). Pyroacm resin **3a** was subjected to trans-thioetherification by using 3.0 equiv of **4a^1** under acidic conditions. Optimal conditions for this process (Table S2), which yields thioether **5a^1**, were found to be 15 vol % of TFA in CH_2Cl_2 for 2 h (Scheme 2). UV spectroscopic analysis of the fluorenylmethyl derivative of **5a^1**, generated by Fmoc cleavage (20% piperidine in DMF), indicates that the resin loading is approximate 0.85 mmol/g. The optimal conditions were used to link **4a^2** and **4b** to **3a**, processes which lead to formation of **5a^2** and **5b**, respectively. In the case of **5b**, the extent of loading was determined using quantitative Ninhydrin analysis.

Introduction of the cysteine residue on the Pyroacm resin under acidic condition minimizes the risk of racemization at the C-terminal residue. However, in the Fmoc protocol for SPPS, repetitive treatment with a 20 vol % piperidine may induce racemization of the cysteine residue. To examine the extent of racemization occurring at the cysteine residue, Pyroacm resins containing linked model tripeptide **7a^1** and **7a^2** were prepared by reactions of **3a** with the D and L isomers of Fmoc-Cys(Trt)-OAllyl and Boc-L-Cys(Trt)-OAllyl, respectively, using Fmoc and Boc protocols (Scheme 3). Sequential removal of the allyl

Scheme 3. Model Tripeptide Synthesis Utilizing the Pyroacm Resin and Its Racemization Studies



and N-protecting groups gave **7a^1** and **7a^2**. Cleavage from the resin to generate tripeptides **8a^1** and **8a^2** was performed by treatment of **7a^1** and **7a^2** with 2 equiv of ScmCl in AcOH. The diastereomers of **8a^1** (Rt = 31.3 min) and **8a^2** (Rt = 33.6 min) can be readily separated using HPLC. Analysis of the chromatograms revealed less than 2% epimerized products **8a^1** and **8a^2** are produced during Fmoc elongation protocol and negligible amount of epimerized product **8a^1**, i.e., <0.4% during Boc protocol (see SI).

To evaluate the stability of Pyroacm resin, **6a^1** (Scheme 3) was subjected to acidic and basic conditions that are employed in the Fmoc and Boc protocols. The results of ninhydrin tests showed that 35% loss of peptide takes place after 24 h exposure to 20 vol % piperidine (Table S3). In addition, the results of Fmoc quantitative analysis showed that even exposure to various acidic environments for long time periods does not cause significant loss of the resin bound peptide (Table S4).

After demonstrating that the new Pyroacm resin can be employed to synthesize model tripeptides, our attention next

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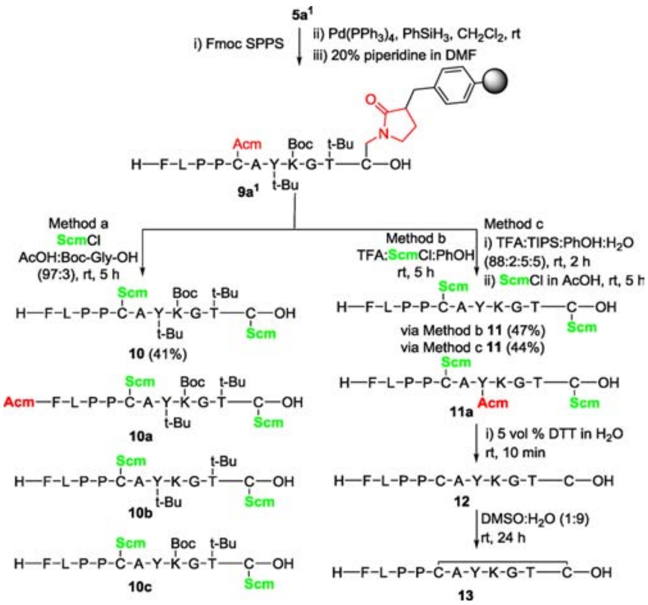
focused on the use of this resin to the preparation of the host-defense skin peptides Riparin 1.2 and Riparin 1.1.[11] For this purpose, the appropriately blocked cysteine containing resin **5a**[1] was transformed to the resin linked undecapeptide **9a**[1] using Fmoc SPPS protocol (Scheme 4).

Scheme 4. Synthesis of Riparin 1.2



The first, **method a** (Table 1), is patterned after the recommended process for conversion of an Acm to a Scm group on a resin (ScmCl in CH_2Cl_2). Under these conditions, **9a**[1] is converted to a mixture containing a major amount of **10a** (Scheme 4), a N-Acm derivative of the target (Table 1, entry 1), but in the LCMS trace of crude reaction, N-Scm substitution has not been observed (Figure S6). Although formation of **10a** in this process was minimized to <3% by using AcOH as an additive or alternative solvent containing ScmCl. But the use of this promoted formation of the Boc-deprotected product **10b** (Scheme 4) containing a free lysine residue of **10** and the *t*-butyl deprotected product **10c** (Scheme 4) containing a free tyrosine residue of **10** (Table 1, entries 2 and 3). Use of 5 vol % MeOH yielded 21% of methyl esterification at C-terminal of **10** (Table 1, entry 4). Finally, by including 3 vol % Boc-Gly-OH in the reagent mixture to serve as an alternate sacrificial reaction site, **10** is generated in a highly pure crude state (Table 1, entry 5). The second procedure, **method b** (Table 2), can be employed to obtain the side chain deprotected target **11** directly from **9a**[1]. However, we observed that the treatment of **9a**[1] with a TFA:ScmCl mixture in a ratio of 99.75:0.25 (i.e., ca. 4 equiv of ScmCl) gave rise to **11a** (Scheme 4), containing Acm substitution at the aromatic ring of tyrosine residue in 26% (Table 2, entry 1).[12] Addition of scavangers, such as 5 vol % TIPS or 5 vol % H_2O, to the reagent mixture was not helpful (29% and 20% of **11a**, respectively, in Table 2, entries 3 and 2). Finally, inclusion of 5 vol % phenol as a scavenger suppressed the production of **11a** to less than 1% and generated **11** in a highly pure crude state (Table 2, entry 4). Surprisingly, the presence of H_2O had no effect on the yield of **11**, but the presence of 2 vol % TIPS leads to a considerable decrease in the overall yield (Table 2, entries 5 and 6) which indicates that ScmCl is quenched by TIPS.

Table 1. Method a To Obtain Side Chain Protected Disulfenyl Riparin 1.2 (10)

entry	reagent	percentage (%)[a] 10	10a	10b	10c	yield of 10 (%)[b]
1	CH_2Cl_2	34	49	2	1	ND
2	CH_2Cl_2:AcOH (9:1)	63	21	6	4	11
3	AcOH	63	2	15	14	23
4[c]	CH_2Cl_2:MeOH (95:5)	68	2	4	3	ND
5	**AcOH: Boc-Gly-OH (97:3)**	**87**	**3**	**<1**	**<1**	**41**

[a]All reactions carried out at 0.01 mmol scale. Product and byproducts in the crude written in percentage. [b]Yield based on purified product after preparative HPLC. [c]21% methyl esterification of **10** observed. ND: not determined.

Table 2. Cleavage Cocktail To Obtain Side Chain Deprotected Disulfenyl Riparin 1.2 (11)[a]

entry	reagent	percentage (%)[b] 11	11a	yield of 11 (%)[c]
1	TFA:ScmCl (99.75:0.25)	67	26	ND
2	TFA:H_2O:ScmCl (94.75:5:0.25)	71	20	ND
3	TFA:TIPS:ScmCl (94.75:5:0.25)	65	29	ND
4	**TFA:PhOH:ScmCl** (94.75:5:0.25)	**92.7**	**0.8**	**47**
5	TFA:PhOH:H_2O:ScmCl (89.75:5:5:0.25)	91.2	1.2	46
6	TFA:PhOH:H_2O:TIPS:ScmCl (87.75:5:5:2:0.25)	88	2.1	09

[a]Reactions carried out at 0.01 mmol scale. Product and byproducts in the crude written in percentage. [b]Yield based on purified product after preparative HPLC. ND: not determined.

Finally, **method c**, involves treatment with TFA:TIPS:PhOH:H_2O (88:2:5:5), i.e., reagent B for 2 h prior to addition of 4 equiv of ScmCl in AcOH. This process cleanly forms the desired product **11** (Scheme 4). In addition, the crude **11** in 5 vol % of DTT in H_2O gave a crude product mixture containing **12** and **13** in a ratio of 95:5. Subjection of the mixture to HPLC purification and treatment of the separated products with 10 vol % DMSO in H_2O for 24 h gave **13**. The crude **13** was purified by HPLC to obtain Riparin 1.2 with high purity (Scheme 4).

Method a, devised in studies of the Riparin 1.2 synthesis, was utilized in the preparation of Riparin 1.1 (Scheme S1) via both the respective Trt and Acm cysteine blocked intermediates, **14a** and **14b**. The cleavage condition using 4 equiv of ScmCl in AcOH was employed to produce the desired product **15** with a high purity in its crude state from both **14a** and **14b**. During cleavage of resin **14a** (Trt) using ScmCl in CH_2Cl_2, no N-Trt transfer took place. Moreover, the use of the reagent combination TFA:ScmCl (99.75:0.25, i.e., 4 equiv of ScmCl) transformed **14b** to **16** in the absence of forming the Acm substituted peptide, an observation that is in agreement with the tyrosine alkylation hypothesis (**11a**) proposed for the case of Riparin 1.2. Finally, use of the optimized conditions

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developed for **9a**[1] was found to promote successful cleavage of both **14a** and **14b** to generate **15** and **16**, respectively. Further, 5 vol % DTT in H_2O reduced *S*-sulfenyl bond of **16** to obtain **17**. The HPLC purified **17** containing 10% aq DMSO required 24−48 h for oxidation to give Riparin 1.1(Scheme S1). All crude reactions HPLC and LCMS have been checked, and purified products subjected to LC MS/MS analysis (see SI).

The new Pyroacm resin, described above, has some noteworthy advantages. It has enabled the synthesis of various C-terminal cysteine containing peptides with high purity. The Pyroacm resin based procedure is compatible with both Fmoc and Boc SPPS. Moreover, in this method the linked C-terminal cysteine does not undergo β-elimination and racemization during Boc protocol. The Pyroacm resin protocol uses inexpensive, commercially available reagents and conditions that are sufficiently mild to be carried out up to a gram scale. During cleavage conditions, method a is applicable to all the amino acid residues; however, methods b and c will give rise to sulfenylation of unprotected Trp residues.[13] Our processes (methods b and c) are still applicable when Trp (CHO) is employed; the CHO group can be cleaved by brief treatment with NaOH after disulfide bond formation to give free Trp.[14] However, further research is needed to apply the Pyroacm resin in broad spectrum.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b00115.

Experimental procedures and characterization data of all reaction via NMR, IR, HPLC, LC/MS, LC MS/MS data (PDF)

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Notes

The authors declare no competing financial interest.

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