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p-Nitromandelic Acid as a Highly Acid-Stable Safety-Catch Linker for Solid-Phase Synthesis of Peptide and Depsipeptide Acids

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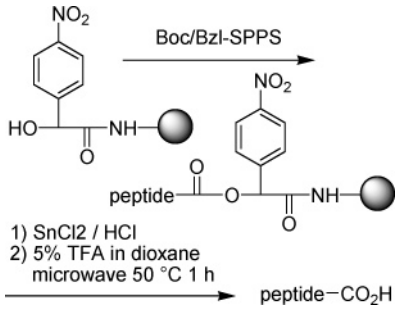
ABSTRACT



***p*-Nitromandelic acid as a safety-catch linker for Boc/Bzl-SPPS of base-labile compounds like peptides and depsipeptides is described. This linker permits acidic removal of side-chain protection groups from the resin. For cleavage from the solid support, the *p*-nitro group was reduced with tin(II) chloride. After washing off the reducing agents, the (depsi)peptide acids with or without the side-chain protection schemes were obtained by microwave irradiation at 50 °C with 5% TFA in dioxane.**

Depsipeptides are naturally occurring compounds that show a broad range of biological activities.[1] They are often cyclic compounds of relatively small ring sizes (5−12-membered). As for cyclic peptides, synthesis of the linear chain on solid support and cyclization after cleavage is a common strategy for their preparation in small-scale runs. However, the choice between the two main SPPS strategies, the Fmoc/^{t}Bu and Boc/Bzl routes, respectively, requires a number of considerations. Ester bonds are base-sensitive and consequently show limited stability toward Fmoc-removal conditions. Thus, there is a risk of continuous loss of ester product if consecutive coupling cycles involving Fmoc-removal are performed after ester bond formation. However, ester bonds are stable toward the acidic conditions of the Boc/Bzl strategy. Consequently, for the synthesis of long linear depsipeptides, the Boc/Bzl route is clearly favored.[2] After cleavage from the solid support with anhydrous HF, the product can be efficiently separated by precipitation from the cleavage cocktail, which normally consists of low-volatile

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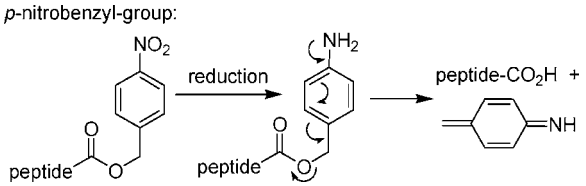
scavengers (anisole, thioanisole, etc.) or, in case of using trifluoromethane sulfonic acid (TFMSA), also nonvolatile strong acid. Unfortunately, precipitation of small (depsi)-peptides may be incomplete and therefore isolation then requires tedious purification procedures. Moreover, the use of conventional Boc/Bzl-protected amino acid building blocks rules out the preparation of the fully side-chain protected peptides required for convergent synthesis or head-to-tail cyclization.

With the aim to overcome the difficulties associated with the synthesis of small depsipeptides, we searched for a linker compatible with the Boc route, which also allows prior removal of the side-chain protection schemes from the resin or cleavage of the fully protected segments, respectively. Safety-catch linkers can meet these challenges. The safety-catch principle involves the conversion of a relatively stable form of a linker into a labile, isolable and cleavable one.[3] However, safety-catch linkers are designed for the Fmoc/*t*-Bu-route are not sufficiently acid-stable. The requirement of nucleophiles or basic conditions for final cleavage after switching on the activity of the linker-bond carbonyl group is shared by a broad range of safety-catch linkers that can be classified as "hidden active esters". We considered this strategy to be less suitable for our purpose because the ester bonds of depsipeptides are sensitive to bases and nucleophiles.

The recently described safety-catch linkers based on the substructure 4-mercapto benzylalcohol are, in their oxidized (sulfinyl) form, stable toward acids and bases. Final cleavage of the peptide is performed by reductive acidolysis.[4a,b] However, under these conditions, benzyl-type protecting groups used in the Boc/Bzl-SPPS are also cleaved.[4c] It is particulary difficult to remove Bzl-type side-chain protecting groups while the peptide is anchored on the resin. *S*-Alkylation occurred in the presence of the scavengers anisole and *p*-cresol and prevents future cleavage from the resin. In contrast, the scavengers thioanisole and thiophenol reduced the linker and caused premature cleavage.[4b]

We studied the potential of *p*-nitromandelic acid (Pnm) as a safety-catch linker. This linker is structurally related to the *p*-nitrobenzyloxycarbonyl (*p*NZ) and *p*-nitrobenzyl groups.[5] This protecting group was, e.g., used for *C*-terminal protection in side-chain anchoring techniques for the SPPS of head-to-tail cyclic peptides.[6] Reduction of the *p*-nitro group of *p*NZ can be performed orthogonally to the Fmoc, Boc, and Alloc protection on the solid phase and causes spontaneous

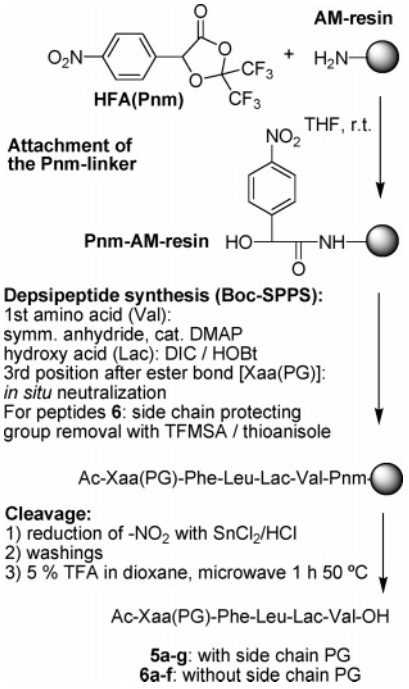
Scheme 1



deprotection of amino and hydroxy groups via 1,6-electron pair shift (Scheme 1).[7] *p*-Nitromandelic acid bears an additional carboxylic group, which allows anchoring on a resin. The (depsi)peptide chain can then be constructed on the α-OH-function. Reduction of the *p*-nitro group would give a *p*-aminomandelic (Pam)-bound peptide, which should be cleaved analogously from the quinonimine methide generated by a 1,6-electron pair shift.

Racemic Pnm was prepared from *p*-nitrobenzaldehyde[8] and protected with hexafluoroacetone (HFA).[9] HFA(Pnm) was then coupled to aminomethylated polystyrene (AM-resin, 1.1 mmol/g) (Scheme 2). The filtrate with the excess of HFA-

Scheme 2



(Pnm) can be reused for further couplings.[9b] Fmoc-Leu-OH was attached as symmetric anhydride in the presence of cat. DMAP to the free hydroxy-function of HO-Pnm-AM to give

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Fmoc-Leu-Pnm-AM-resin **1**. For the synthesis of Fmoc-Ile-Phe-Leu-Pnm-AM-resin **2**, Boc-Leu-OH was attached first and the following amino acids were coupled with DIC/HOBt activation. The drastic decrease in resin loading to 0.11 mmol/g demonstrates the high propensity to form diketopiperazine, which was circumvented in further peptide synthesis by the in situ neutralization method for couplings of amino acid in the third position after the ester bond.[10]

The *p*-nitro function of Pnm in **1** and **2** was then reduced using the same conditions applied for reduction of the *p*NZ-group (6 M $SnCl_2$/1.6 mM HCl/dioxane-solution in DMF for 1 h).[7] Two intensive IR-signals associated with aryl-bound nitro-groups at 1524 and 1347 cm^{-1} disappeared. In comparison with the rapid cleavage of the *p*NZ group, which proceeds spontaneously after reduction, the resin-bound depsipeptides **3** and **4** with *C*-terminal *p*-aminomandelic acid (Pam) were relatively stable. The loss of peptide or amino acid during reduction and subsequent washings of the resin was in the range of 1.3−23.4%, with an average of 12.2% (10 experiments).[11] This stability enabled the removal of most of the tin salt excess.[12]

Next, we tested several conditions to cleave the amino acid or peptide from Pam-AM resin. Short treatment with 10% DIEA in DCM showed only a small effect (Table 1, entry 1) and prolonged treatment caused, as expected, an unselective ester bond rupture (Table 1, entry 2 vs 3). The resin-bound peptide was also relatively stable in organic solvents at room temperature (entry 4), but the amount of cleaved product increased by heating, which was performed in a controlled manner by microwave irradiation (entries 5 and 6). Better results were achieved with 90% TFA (entries 7 and 8). However, prolonged treatment up to 72 h did not lead to complete cleavage of product (entry 9). Combining acidic media (5% TFA in dioxane) with 1 h heating to 50 °C gave the best results (entry 10) and was found to be selective for the reduced form of the linker, because from the unreduced resin **2** practically no product was cleaved (Table 1, entry 11).

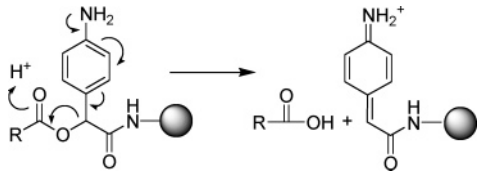
Table 1. Cleavage Conditions

entry	resin[a]	conditions	% cleavage (% remaining on resin)[b]
1	**3**	3 × 2 min, 10% DIEA in DCM	5 (77)
2	**4**	10% DIEA in DCM, overnight	50 (44)
3	**2**	10% DIEA in DCM, overnight	27
4	**3**	DMF, overnight	33
5	**4**	DMF, MW 50 °C, 1 h	75 (9)
6	**4**	THF, MW 50 °C, 1 h	65 (22)
7	**3**	TFA/DCM/H_2O (90:5:5), overnight	58 (19)
8	**4**	TFA/DCM/ H_20 (90:5:5), overnight	56
9	**3**	TFA/DCM/ H_20 (90:5:5), 72 h	66 (22)
10	**4**	TFA/dioxane (5:95), MW 50 °C, 1 h	85 (12)
11	**2**	TFA/dioxane (5:95), MW 50 °C, 1 h	99[c]

[a] Unreduced form of the linker: **1**, Fmoc-Leu-*Pnm*-AM; **2**, Fmoc-Ile-Phe-Leu-*Pnm*-AM. Reduced form of the linker: **3**, Fmoc-Leu-*Pam*-AM; **4**, Fmoc-Ile-Phe-Leu-*Pam*-AM. [b] Quantified by Fmoc determination measuring UV absorbance at 290 nm. [c] Colored solution.

The quinonimine methide generated from the 1,6-electron shift can also react with nucleophiles, which explains why TFA cleaved the Pam-bound depsipeptides more efficiently than DIEA. This principle was recently used in the design of activity-based fluorescent probes that target proteases.[13] We propose that, in our case, the quinonimine methide and the *C*-terminal carboxylic anion of the peptide recombined quickly under neutral conditions or in the presence of DIEA. However, under acidic conditions, the carboxylic acid, which acts as leaving group, is formed. This would explain the better cleavage rates in acidic medium (Scheme 3).

Scheme 3



By means of Boc/Bzl-SPPS standard procedures, we then prepared a number of 5-membered depsipeptides containing at least one amino acid with an additional functionality bearing the common benzylic side-chain protecting group (Scheme 2). To avoid diketopiperazine formation, the second position after the ester bond was a hydroxy acid (lactic acid, Lac) and an amino acid was introduced on fifth position by in situ neutralization.[10] To remove the side chain protecting groups, a part of each resin was treated with TFMSA in the presence of thioanisole as scavenger. These reagents were removed from the resin by filtration and washing. After reduction of the *p*-nitro group, the depsipeptide was cleaved under the conditions described above (5% TFA in dioxane at 50 °C, microwave heating). The solutions were evaporated, and the residues redissolved in water/acetonitrile and lyophilized. The main peaks in the HPLC spectra were the desired products (HPLC-MS, ESI). Table 2 shows the yield and the purity of the protected (**5a**−**g**) and unprotected (**6a-f**) depsipeptides obtained. For side-chain unprotected Cys-depsipeptide **6e** dimerization decreased the yield. A lower

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(11) Calculated as the difference between inital loading and the sum of product remaining after reduction on the resin and cleaved product (for conditions, see Table 1) because direct determination of UV absorbance of tin-containing solutions is not credible due to yellow color and precipitates.

(12) The amount of tin was found to be 20 ppm for a product washed with water in a Diaion (Supelco) column as determined by ICP (inductively-coupled plasma).

(13) A peptide recognition sequence was attached to the *p*-amino group of Pam, a fluorescence reporter was linked to the carboxy group, and the 2-hydroxy group was exchanged for fluorine as leaving group. After cleavage of the peptide by the protease, the fluorine is released and the quinonimine methide reacts with a nucleophilic site present in the enzyme to form a covalent bond with the fluorescence-reporter system. Srinivasan, R.; Huang, X.; Ng, S. L.; Yao, S. Q. *ChemBioChem* **2006**, *7*, 32−36.

Table 2. Yields and Purities of Cleaved Products

product (Xaa)[a]	amount in mg (from mg of resin)	% HPLC purity (retention time in min)
5a [Glu(OBzl)	26 (80)	76 (7.40[b])
6a (Glu)	9.5 (39)	82 (5.90[c])
5b [Lys(2ClZ)]	25 (90)	78 (8.03[b])
6b (Lys)	8 (40)	75 (7.95[d])
5c [Thr(Bzl)]	19.5 (90)	77 (7.79[b])
6c (Thr)	9.5 (40)	66 (6.02[c])
5d [Tyr(Bzl)]	22.5 (90)	72 (9.07[b])
6d (Tyr)	10 (40)	77 (7.23[c])
5e [Cys(pMeBzl)]	21 (85)	73 (8.45[b]); 5 (oxidized product) (6.19[b])
6e (Cys)	4 (35)	18 (10.45[d]); 32 dimer (11.91,[d] 1186.3)
5f [Arg(Tos)]	10.5 (50)	72 (5.46[b])
6f (Arg)	4.5 (20)	72 (6.91[d])
5g [Asn(Xan)]	7 (80)	53 (without Xan) (10.49[e])

[a] See Scheme 2. [b] Gradient 40−100% ACN, 15 min. [c] Gradient 30−100% ACN, 15 min. [d] Gradient 20−100% ACN, 15 min. [e] Gradient 0−100% ACN, 15 min.

yield was also observed for Asn **5g**. However, the other depsipeptides were obtained in reasonable purity and yields.

In summary, we have demonstrated that *p*-nitromandelic acid can be used as a highly acid-stable safety-catch linker, compatible with the Boc/Bzl strategy for the synthesis of peptides and depsipeptides. Cleavage of the final products can be performed without base. Therefore, on the basis of these observations, we propose that this linker is suitable for future applications especially for the synthesis of base-labile compounds on the solid phase.

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Supporting Information Available: Synthesis and NMR-spectroscopic data of HFA-*p*-nitromandelic acid, protocols used for peptide synthesis, details of HPLC-measurements. This material is available free of charge via the Internet at http://pubs.acs.org.

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