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The Phenyl Hydrazide as an Enzyme-labile Protecting Group - Oxidative Cleavage with Mushroom Tyrosinase

Gernot H. Müller and Herbert Waldmann*

Universität Karlsruhe, Institut für Organische Chemie, Richard-Willstätter-Allee 2, D-76128 Karlsruhe, Germany

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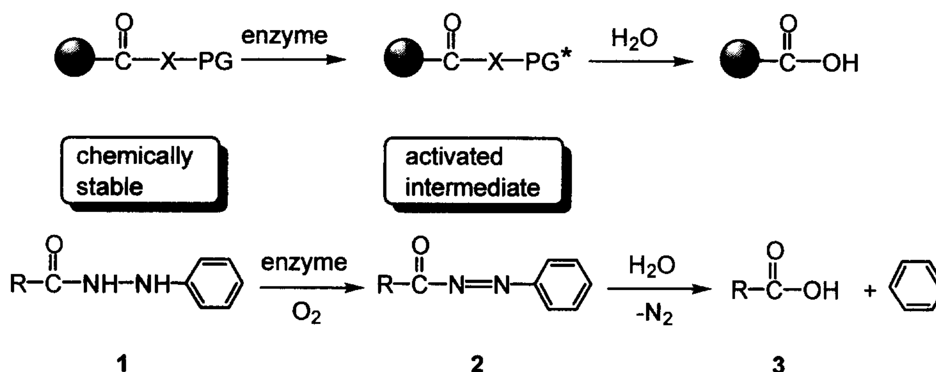
Abstract: Amino acid and peptide phenyl hydrazides are selectively cleaved by oxidation to acyl diazenes with mushroom tyrosinase and their subsequent hydrolysis. © 1999 Elsevier Science Ltd. All rights reserved.

The controlled and highly selective introduction and removal of protecting groups belongs to the most frequently carried out transformations in many areas of organic synthesis, in particular in the construction of natural products.[1] Therefore, the development of new and alternative protecting group techniques is of great interest. For this purpose the application of enzymatic transformations has yielded valuable alternatives to classical chemical methods.[2] In particular, enzymatic protecting group techniques were successfully applied in the synthesis of peptide conjugates and carbohydrates.[3] Usually hydrolases directly attacking carbonyl groups are employed to remove protecting groups, and the substrate specificity of the individual enzymes guarantees the required chemo- and regioselectivity. An alternative approach, however, might consist in applying an enzymatic transformation that converts a chemically stable precursor substrate into a labile intermediate followed by non-enzymatic hydrolysis (Scheme 1).

Scheme 1



By analogy to classical chemical approaches such an enzyme-labile „two-step protecting group"[1] could for instance employ oxidation or reduction reactions and would, therefore, be orthogonally stable to hydrolase-sensitive blocking functions.[4] Phenyl hydrazides **1** can be converted to carboxylic acids **3** by such a two-step process that involves enzyme-mediated oxidation of the hydrazide **1** to an acyl diazene **2** and its subsequent

Fax: Intl.(+)721/608-4825 - e-mail: waldmann@ochades.chemie.uni-karlsruhe.de

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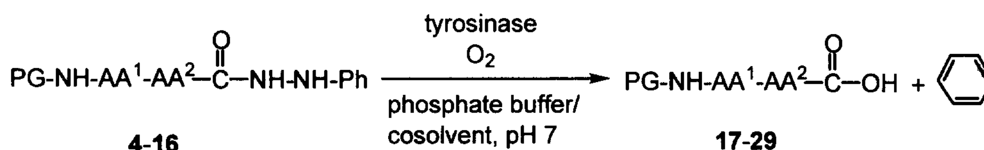
hydrolysis[5] (Scheme 1). Unfortunately, the enzymes described as being able to catalyze this process (horseradish peroxidase and laccase from *Coriolus hirsutus*) are sensitive to inhibition by organic cosolvents (horseradish peroxidase) or not readily available. Consequently, the enzymatic cleavage of phenyl hydrazides has not found further use in organic synthesis. In an attempt to identify an alternative readily available biocatalyst capable of inducing the two-step cleavage of the phenyl hydrazide protecting group we investigated the use of mushroom tyrosinase. Like laccase this enzyme has cresolase activity and we speculated that it might be able to oxidize hydrazides to acyl diazenes.

Tyrosinase is commercially available (Sigma) and alternatively it can readily be isolated via a simple procedure from the regular commercially available mushrooms *Agaricus bisporus* (Champignons). It tolerates the presence of up to 30 vol % of organic cosolvents like acetonitrile, dioxane or DMF and operates even in organic media.[7]

In a series of orientating experiments Boc- and Z-protected amino acid phenyl hydrazides **4** - **12** were treated with tyrosinase (preparation 1[6]) in buffer/cosolvent mixtures at pH 7. The solutions rapidly turned red due to formation of the diazenes and the desired Boc- and Z-protected amino acids were liberated in high yields[8] (Scheme 2; Table 1).

Scheme 2



To obtain a high deprotection rate, O_2 should be bubbled through the solution, otherwise the initial oxidation reaction is slowed down (Table 1, compare entries 1 and 2 with entries 3 - 9). Also, the substrate should be solubilized by addition of a sufficient amount of organic cosolvent. Whereas in the majority of the cases in the presence of 10 - 15 vol % of acetonitrile a rapid deprotection occurred, Boc- protected phenylalanine hydrazide **8** and Z-protected substrates **11** and **12** were not attacked under these conditions. In the case of **8** the problem could be overcome by sonication of the solution (Table 1, entry 5) and for leucine hydrazide **11** the use of DMF instead of acetonitrile was efficient (Table 1, entry 8). Z-protected phenylalanine hydrazide **12** could not be solubilized under these conditions and was not deprotected (Table 1, entry 9).

In order to determine if mushroom tyrosinase can also remove the phenyl hydrazide protecting group selectively from peptides, Boc- and Z-protected peptide phenyl hydrazides **13** - **16** were prepared and subjected to the enzyme-initiated deprotection reaction. Initial experiments revealed that enzyme preparation 1[6] used for the reactions detailed above contained still trace amounts of a protease that attacked the peptide bonds. Therefore, tyrosinase was further purified by hydrophobic interaction chromatography on phenyl sepharose.[6] The purified enzyme preparation 2 obtained thereby was no longer contaminated with protease activity.

Upon treatment of peptides **13** - **16** with this tyrosinase preparation in phosphate buffer at pH 7[7] the C-terminal protecting group was cleaved rapidly and without any undesired side reaction. Neither the peptide bonds nor the N-terminal urethane group were attacked and the selectively unmasked peptides were isolated in high yields (Table 1, entries 10 - 13). Particularly remarkable is the observation that tyrosinase attacks peptides **15** and **16** carrying a threonine and a proline at the C-terminus, respectively. This is in remarkable contrast to the finding that peptide prolyl esters are not attacked by lipases at all or only with low yields.[9] Also, the lipase-mediated deprotection of threonyl peptides often is problematic.[3c,10] Thus, the use of tyrosinase offers an advantageous solution to these problems.

In conclusion the phenyl hydrazide protecting group can be cleaved with mushroom tyrosinase in an enzyme-initiated two step process (two-step protecting group). The enzymatic transformation proceeds with complete selectivity, i. e. neither the N-terminal protecting groups nor the peptide bonds are attacked. The conditions for the removal of this enzyme-labile blocking function are so mild that this protecting group

technique should serve well in the construction of acid- and base-labile peptide conjugates like lipo-, glyco-, phospho- and nucleopeptides.

Table 1: Results of the tyrosinase-catalyzed cleavage of Boc- and Z-amino acid and peptide phenyl hydrazides.

entry	no	PG	AA[1]	AA[2]	isolation method[8]	cosolvent/ vol %	reaction time [h]	yield [%][a]
1	**4**	Boc	Gly	-	B	CH_3CN/10	72[b]	88
2	**5**	Boc	Ala	-	B	CH_3CN/10	216[b]	89
3	**6**	Boc	Val	-	A	CH_3CN/10	16	99
4	**7**	Boc	Leu	-	A	CH_3CN/10	16	89
5	**8**	Boc	Phe	-	A	-[c]	6	65
6	**9**	Z	Gly	-	A	CH_3CN/15	5	94
7	**10**	Z	Ala	-	B	CH_3CN/10	5	70
8	**11**	Z	Leu	-	A	DMF/20	16	88
9	**12**	Z	Phe	-	-	DMF/20	-	-
10	**13**	Boc	Val	Ala	A	CH_3CN/15	16	90
11	**14**	Z	Leu	Ser	A	dioxane/20	4	83
12	**15**	Boc	Leu	Thr	A	CH_3CN/10	5	94
13	**16**	Z	Ala	Pro	A	CH_3CN/5	6	66

[a] For chromatographically purified compounds. All products were identified by ^{1}H- and ^{13}C-NMR spectroscopy; [b]without bubbling O_2 through the solution; [c] enzyme preparation 2[6] was used; the solution was sonicated.

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4. We note, however, that also hydrolase-initiated reactions may be used to develop enzyme-labile „two-step protecting groups“. For urethanes that are cleaved by enzymatic hydrolysis of an adjacent ester and subsequent non-enzymatic fragmentation of an intermediate phenolate see: a) E. Nägele, M. Schelhaas, N. Kuder, H. Waldmann, *J. Am. Chem. Soc.* **1998**, *120*, 6889-6902., b) T. Pohl, H. Waldmann, *J. Am. Chem. Soc.* **1997**, *119*, 6702-6710
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6. Fresh mushrooms (3 kg) obtained from a local cultivation company were frozen with liquid nitrogen, homogenized in acetone (5.5 l) using a Waring blender and centrifuged at 7000 rpm for 20 min. The pellet was treated with liquid nitrogen and then suspended in 2 l of phosphate buffer (pH 7, 0.1 M, treated with 20 g of polyvinyl pyrrolidone two hours before use). The mixture was kept overnight at 4°C while nitrogen was bubbled gently through to remove remaining acetone. The suspension was centrifuged for 40 min at 10000 rpm and the pellet was discarded. After addition of 176 g of $(NH_4)_2SO_4$ per liter solution and 30 min equilibration at 0°C (30% saturation) the mixture was centrifuged (10000 rpm, 40 min) and the pellet was discarded.

   Preparation 1: After addition of 195 g of $(NH_4)_2SO_4$ per liter solution and 30 min equilibration at 0°C (60% saturation) the mixture was centrifuged (8000 rpm, 40 min). The pellet was dissolved in 520 ml of distilled water, dialyzed against distilled water and lyophilized to yield 7.3 g of a grey powder containing 50000 units of enzyme activity.

   Preparation 2: After addition of 1.66 l of phosphate buffer (pH 7, 0.1 M) to adjust the $(NH_4)_2SO_4$ concentration to 0.7 M the solution was filtered (filter G4) and the filtrate was passed over a column loaded with phenyl sepharose (Pharmacia: Phenyl sepharose HP, column volume 70 ml). After washing with 2 l of phosphate buffer (0.1 M, pH 7) containing 0.65 M $(NH_4)_2SO_4$ and elution with phosphate buffer (0.1 M, pH 7) containing 0.2 M $(NH_4)_2SO_4$ the fractions containing the enzymatic activity were pooled, concentrated to 140 ml with an ultrafiltration system (30 kDa membrane), dialyzed against phosphate buffer (pH 7, 0.05 M) and lyophilized to yield 957 mg of a grey powder containing 17000 units of enzyme activity.

   Both preparations were stored until use at -23°C.

   The activity was assayed using an adapted „dopachrome" method as described.[7] One unit of enzymatic activity is defined as the amount of enzyme which produces 1 mmol of dopachrome/min in a solution of L-DOPA (10 mM in phospate buffer (pH 6.0, 50mM)). The reaction is followed with a spectrophotometer monitoring the increase in absorbance due to dopachrome formation at 475 nm ($\varepsilon = 3600^{*}\ M^{-1}\ cm^{-1}$).

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8. To a solution of 50 - 100 mg of protected amino acid or peptide in the corresponding amount of cosolvent 40 ml of phosphate buffer was added. After saturation with O_2 (3 min) the mixture was treated with 100 - 340 mg of tyrosinase (preparation 1) dissolved in buffer (total volume 100 - 250 l). O_2 was bubbled through the reaction mixture. When TLC indicated cleavage was complete the cosolvent was distilled off in vacuo. Isolation method A: The remaining liquid was lyophilized. The residue was suspended in 20 ml of methanol, sonicated (1 min) and filtered through Celite. After evaporation of the solvent in vacuo the residue was purified by flash chromatography on silica gel (eluent: hexane/ethyl acetate mixtures with 1 % acetic acid). Isolation method B: to the aqueous solution HCl was added until the pH reached 1 - 2. The product was extracted with ethyl acetate and after evaporation of the solvent in vacuo the residue was purified by flash chromatography on silica gel (eluent: hexane/ethyl acetate mixtures with 1 % acetic acid).

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