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Phenylhydrazide as an Enzyme-Labile Protecting Group in Peptide Synthesis

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The enzymatic cleavage of amino acid phenylhydrazides with the enzyme tyrosinase (EC 1.14.18.1) offers a new, mild, and selective method for C-terminal deprotection of peptides. The advantages of the described methodology are the very mild oxidative removal of the protecting group at room temperature and pH 7, a high chemo- and regioselectivity, and the availability of the biocatalyst. Even in oxygen-saturated solution, the oxidation of sensitive methionine residues was not observed. These features make the methodology suitable for the synthesis of sensitive peptide conjugates. Mechanistic data suggest that the hydrolysis of the oxidized adducts proceeds by a free-radical mechanism.

Introduction[1]

During the past decades, it has become evident that the proper function of many proteins depends on post-translational modifications. To reveal the biological role of these modifications, chemically synthesized modified peptides embodying the characteristic structural units of the modified protein have proven to be invaluable items in the toolbox of chemical biology.[2-4] But due to the distinct sensitivy of such peptide conjugates the synthesis of a specific target compound may be a formidable challenge often not executable with the existing technologies and, thus, calling for the development of new synthesis methods.

In particular, in the synthesis of peptide conjugates such as glyco-, phospho-, nucleo-, and lipidated peptides the pronounced acid and base labilities of these target molecules have to be taken into account.[5] The chemistry of these compound classes requires that particularly mildly removable protecting groups are available to the organic chemist. The use of enzyme-labile protecting groups may offer viable alternatives to classical methodologies. Enzymes combine a high selectivity for the recognized structure with a broad substrate tolerance, and additionally they mostly operate at very mild conditions, i.e., near neutral pH and in a temperature range from 0 to 50 °C.[6] Enzyme-labile protecting groups for carboxy and amino functions have been developed and successfully applied in the synthesis of lipo-,[7] phospho-,[8] glyco-,[9,10] and nucleopeptides.[11-13] Even in solid-phase synthesis, the use of enzymes is possible using enzyme-labile linker groups.[14] Most commonly, the applied enzymes are hydrolases that directly attack carbonyl groups of esters and/or amides. Here, the substrate specificity of the indiviual enzyme, in particular the possibility of undesired peptide bond hydrolysis, has to be taken into account to guarantee the required chemo- and regioselectivity.

An alternative approach is the use of a different enzymatic transformation in a two-step-procedure during which an otherwise stable precursor is converted enzy-

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(1) Abbreviations used: HOBt, 1-hydroxybenzotriazole; TFA, trifluoroacetic acid; DIPEA, diisopropyl ethylamine; DCM, CH_2Cl_2; PalCl, palmitoyl chloride.

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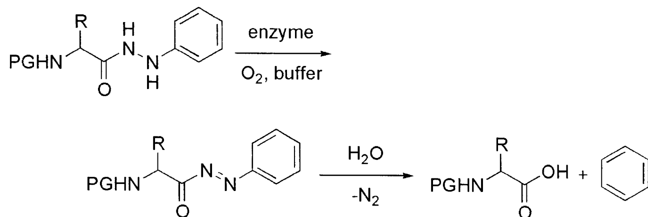
SCHEME 1. Enzymatic Cleavage of Amino Acid Phenylhydrazides



matically into a labile intermediate that subsequently hydrolyzes spontaneously under the reaction conditions. Such a transformation could be an oxidation reaction, which would—if designed appropriately—allow for orthogonality to different hydrolase-labile protecting groups and to other classical chemical blocking groups. This paper describes the use of the amino acid phenylhydrazide as such an enzyme labile C-terminal two-step blocking function (Scheme 1).[15]

The oxidation of N-terminally protected amino acid phenylhydrazides to diimines has previously been used for acylation reactions, e.g., in the synthesis of peptides.[16−19] The oxidation can be performed using *N*-bromosuccinimide (NBS) or alternatively with Cu salts or MnO_2, but only in the NBS protocol can the intermediary formed acyl diazenes be isolated. It was further reported that the phenylhydrazides could be enzymatically oxidized by the oxido-reductases laccase and horseradish peroxidase.[20] Unfortunately, laccase is sensitive to inhibition by organic cosolvents, and therefore, its use is restricted to substrates that are readily soluble in aqueous buffer. To overcome this problem, we investigated the applicability of mushroom tyrosinase in this reaction.

Tyrosinase (EC 1.14.18.1) occurs in many organisms and is, for instance, involved in the browning of fruits and plants, in insect exoskeleton formation, and in melanin pigmentation in vertebrates.[21,22] The enzyme catalyzes the *o*-hydroxylation of monophenols (cresolase activity) and the oxidation of *o*-diphenols to *o*-quinones (catecholase activity). The active site of tyrosinase embodies two copper atoms that in the deoxy-Cu(I)−Cu(I) form can reversibly bind oxygen.[22,23]

Tyrosinase is commercially available from Sigma, and alternatively, it can be isolated from common mushrooms (*Agaricus bisporus*) by means of a simple procedure.[15,24] This procedure gives access to large quantities of the biocatalyst at very low cost. The enzyme tolerates the presence of up to 30 vol % of organic cosolvents such as acetonitrile, dioxane, or DMF and even operates in organic media.[25−27]

SCHEME 2. Introduction of the Phenylhydrazide Moiety

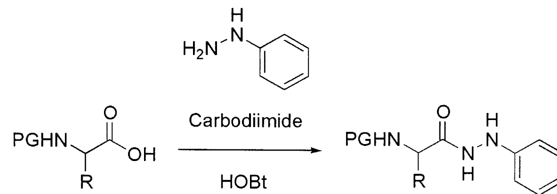


TABLE 1. Examples for the Protection of Amino Acids as Phenylhydrazides

entry	amino acid	carbodiimide	yield (%)
1	Z-Gly-NHNHPh **1**	DCC	81
2	Z-Ala-NHNHPh **2**	DCC	56
3	Boc-Gly-NHNHPh **3**	EDCI	84
4	Boc-Val-NHNHPh **4**	DCC	99
5	Boc-Leu-NHNHPh **5**	DIC	91
6	Boc-Pro-NHNHPh **6**	EDCI	96
7	$(Boc\text{-}Cys\text{-}NHNHPh)_2$ **7**	EDCI	92
8	Fmoc-Gly-NHNHPh **8**	DIC	64
9	Fmoc-Pro-NHNHPh **9**	EDCI	71

TABLE 2. Examples for the *N*-Terminal Deprotection of Amino Acid Phenylhydrazides

entry	amino acid	cleavage reagent	yield (%)
1	Z-Ala-NHNHPh **2**	H_2, Pd/C	92
2	Boc-Val-NHNHPh **4**	TFA	91
3	Boc-Leu-NHNHPh **5**	TFA	99
4	Boc-Pro-NHNHPh **6**	TFA	93
5	Fmoc-Pro-NHNHPh **9**	piperidine	92

Results and Discussion

Synthesis of Substrates. The phenylhydrazide moiety was introduced into N-terminally masked amino acids in good to excellent yields using a carbodiimide-mediated coupling methodology (Scheme 2, Table 1).

Boc, Z, and Fmoc were applied as N-terminal protecting groups, and dicyclohexylcarbodiimide (DCC), diisopropylcarbodiimide (DIC), and *N*(3-dimethylaminopropyl)-*N*-ethylcarbodiimide (EDCI) were used together with HOBt for carboxyl activation. With Boc and Z, a slight excess of phenylhydrazine can be used while this is not favorable for Fmoc-amino acids to avoid undesired cleavage of the Fmoc group.

After formation of the phenylhydrazide, the N-terminal protecting group was removed selectively for subsequent elongation by the known methods. Results for the unmasking are given in Table 2. The phenylhydrazide group turned out to be compatible with the three protecting groups, i.e., it is stable to hydrogenolysis, TFA and treatment with piperidine.

Peptide chain elongation to dipeptides was achieved via carbodiimide-mediated peptide coupling. As a repre-

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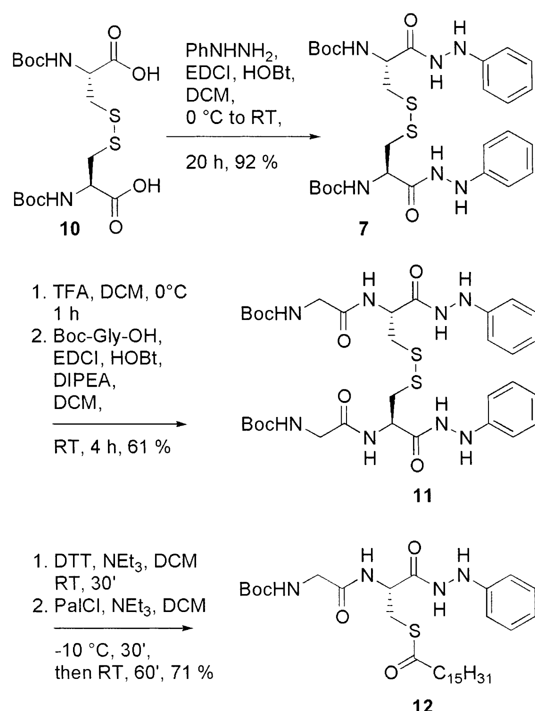
TABLE 3. Yields and Conditions for the Cleavage of Masked Amino Acid Phenylhydrazides with Tyrosinase

entry	compound	cosolvent	vol (%)	time (h)	mass equiv enzyme	convn[a] (%)	yield[b] (%)
1	Boc-Gly-NHNHPh **3**	MeCN	10	72[c]	2.5	quant	88
2	Boc-Gly-NHNHPh **3**	MeCN	10	17	0.2	46	74
3	Boc-Ala-NHNHPh **13**	MeCN	10	216[c]	3.4	quant	89
4	Boc-Ala-NHNHPh **13**	MeCN	10	23	0.2	45	55
5	Boc-Val-NHNHPh **4**	MeCN	10	16	2.5	quant	99
6	Boc-Leu-NHNHPh **5**	MeCN	10	16	2.5	quant	89
7	Boc-Phe-NHNHPh **14**	*d*		6	2.0	quant	65
8	Boc-Phe-NHNHPh **14**	MeCN	10	15	0.2	60	87
9	Boc-Trp-NHNHPh **15**	MeCN	10	24	0.2	58	75
10	Boc-His(Bzl)-NHNHPh **16**	MeCN	10	42	0.2	30	13
11	Fmoc-Pro-NHNHPh **9**	MeCN	10	192	0.42	22	58
12	Aloc-Ser-NHNHPh **17**	MeCN	10	16	2.5	quant	52
13	Z-Gly-NHNHPh **1**	MeCN	15	5	2.5	quant	94
14	Z-Ala-NHNHPh **2**	MeCN	10	5	2.0	quant	70
15	Z-Leu-NHNHPh **18**	DMF	20	16	2.5	quant	88

[a] Conversion calculated from isolated starting material. [b] Yield based on consumed starting material (see text for an example). [c] Without O_2-bubbling. [d] Ultrasonication.

SCHEME 3. Synthesis of Boc-Gly-Cys(Pal)-NHNHPh (12)



sentative example, the synthesis of the palmitoylated dipeptide Boc-Gly-Cys(Pal)-NHNHPh (**12**) is depicted in Scheme 3.

Boc-L-cystine **10** was converted to phenylhydrazide **7** by the method described above. After N-terminal elongation, the disulfide bond of **11** was cleaved with dithiothreitol (DTT), followed by palmitoylation at −10 °C. When the acylation procedure was carried out at room temperature, additional acylation at a phenylhydrazide N-atom was observed. The palmitoylated phenylhydrazide **12** could be synthesized by this sequence in 41% overall yield.

The synthesis of the other dipeptides proceeded uneventfully in a likewise manner, typically yielding between 60 and 80% of the desired dipeptides.

Selective Cleavage of the Phenylhydrazide. For the cleavage of the phenylhydrazide group, the protected amino acids and peptides were treated on 50−100 mg scale at room temperature with tyrosinase in phosphate buffer and cosolvents at pH 7.0. In Table 3, an overview of the substrates and conditions employed is given. For screening purposes, 0.2 mass equiv of enzyme were used. However, since the conversion rate can be increased by using more enzyme, in preparative applications the amount of enzyme was increased to speed up the reaction. The degree of conversion given in Table 3 was calculated from the reisolated unconsumed starting material. The yield is given as the amount of isolated product based on the consumed starting material, i.e., at 80% conversion 72% isolated yield correspond to 90% yield based on consumed starting material.

To maintain a high oxygen concentration in the reaction mixture, it is advisable to perform the reaction under continuous bubbling of oxygen through the solution since oxygen is only poorly soluble in the buffer. When performed under air in an open flask, the reaction time increases drastically (Table 3, entries 1 and 3). Furthermore, the substrate should be solubilized and thus rendered accessible to the biocatalyst by addition of up to 20 vol % of acetonitrile or DMF. Alternatively, also a partial solubilization by ultrasonication (Table 3, entry 7) improves the deprotection reaction. As a general result from these experiments, we conclude that various amino acid phenylhydrazides are cleaved by tyrosinase, including proline derivatives. Tyrosine, of course, would have to be protected at the phenol group. It should be noted that the solubility of the substrate is crucial: the poorly soluble substrates Boc-His(Bzl)-NHNHPh (**16**) and Fmoc-Pro-NHNHPh (**9**) require far longer reaction times than most of the other substrates, but still the conversion and the yield of the isolated product are lower. This should be taken into account in choosing further protecting groups. We have further demonstrated for several examples that by increasing the amount of enzyme the reaction can be driven to full conversion in shorter time and with good to excellent yields. To exclude the possibility of an auto-oxidative cleavage of the phenylhydrazide moiety, the transformations were checked against a blank sample without enzyme in buffer in the presence of oxygen. For none of the reactions was a background cleavage observed.[28]

TABLE 4. Phenylhydrazide Cleavage from Dipeptides with Tyrosinase

entry	compound	cosolvent	vol (%)	time (h)	mass equiv enzyme	convn[a] (%)	yield[b] (%)
1	Boc-Leu-Pro-NHNHPh **19**	MeCN	10	19	0.2	32	48
2	Boc-Gly-Val-NHNHPh **20**	MeCN	10	19	0.2	80	96
3	Boc-Gly-Leu-NHNHPh **21**	MeCN	10	55	0.2	90	83
4	Boc-Gly-Leu-NHNHPh **21** [c]	MeCN	4	44	0.35	95	98
5	Boc-Met-Gly-NHNHPh **22**	MeCN	10	17	0.3	75	89
6	Boc-Met-Gly-NHNHPh **22** [d]	MeCN	10	74	0.45	75	75
7	Boc-Trp-Trp-NHNHPh **23**	MeCN	10	197	0.45	18	85
8	Boc-Trp(Bzl)-Gly-NHNHPh **24**	MeCN	10	108	0.33	40	55
9	Boc-Phe-Phe-NHNHPh **25**	MeCN	10	108	0.33	33	80
10	Fmoc-Phe-Phe-NHNHPh **26**	MeCN	10	108	0.33	56	17
11	Fmoc-Phe-Thr(Bzl)-NHNHPh **27**	MeCN	10	110	0.2	23	70
12	Boc-Gly-Cys(Pal)-NHNHPh **12**	MeCN	10	180	0.45	76	99
13	Boc-Val-Ala-NHNHPh **28**	MeCN	15	16	1[e]	quant	90
14	Z-Leu-Ser-NHNHPh **29**	dioxane	20	4	1[e]	quant	84
15	Boc-Leu-Thr-NHNHPh **30**	MeCN	10	5	1[e]	quant	94
16	Z-Ala-Pro-NHNHPh **31**	MeCN	5	6	1[e]	quant	66

[a] Conversion calculated from isolated starting material. [b] Isolated yield, calculated from converted starting material. [c] 0.5 mmol scale. [d] 1.6 mmol scale. [e] HIC-purified enzyme preparation.

TABLE 5. Enzymatic Removal of the Phenylhydrazide Group from Tetra- and Hexapeptides with Tyrosinase

entry	compound	cosolvent	vol (%)	time (h)	mass equiv enzyme	convn[a] (%)	yield[b] (%)
1	Boc-Met-Gly-Leu-Pro-NHNHPh **38**	MeCN	10	24	0.8	82	86
2	Boc-Met-Gly-Met-Gly-NHNHPh **34**	MeCN	10	24	0.55	90	90
3	Fmoc-Met-Gly-Leu-Pro-Met-Gly-NHNHPh **39**	MeCN	10	94	0.5	39	39
4	Boc-Gly-Leu-Met-Gly-Met-Gly-NHNHPh **37**	MeCN	4	15	0.88	78	73

[a] Conversion calculated from isolated starting material. [b] Isolated yield, calculated from converted starting material.

The applicability of the enzymatic cleavage of the phenylhydrazide was further investigated by the deprotection of a series of dipeptides (Table 4).

In an initial screening experiment, the deprotection of 11 representative dipeptides embodying different amino acids with varying N-terminal and side-chain protecting groups was investigated. In all cases, the phenylhydrazide moiety was cleaved, although again the reactions of the unpolar substrates resulted in lower conversions and yields even after prolonged reaction times (Table 4, entries 7−12). For four substrates (Table 4, entries 13−16), the conditions were optimized to quantitative conversion. For these reactions, an enzyme preparation purified by hydrophobic interaction chromatography (HIC) was used that showed a 2.5-fold higher activity than the otherwise employed enzyme preparation.[15] In all experiments, the reaction took place with high chemoselectivity: in none of the reactions were the peptide bonds or the other protective groups attacked. Remarkably, also the methionine thioether was not oxidized during the reaction. With Boc-Gly-Cys(Pal)-NHNHPh (**12**) (Table 4, entry 12), it could be demonstrated that the methodology is also applicable to the synthesis of base-sensitive peptide conjugates. Neither β-elimination nor hydrolysis of the labile thioester were observed, although in this case again the drawback of the poor solubility led to satisfying conversion only after prolonged reaction time. This is, however, the first example for the deprotection of a palmitoylated peptide conjugate by the enzymatic removal of a phenylhydrazide. It suggests that the phenylhydrazide group may open up new opportunities to the field of lipopeptide- and peptide conjugate synthesis in general.

The cleavage of Boc-Met-Gly-NHNHPh (**22**) was repeated on a 1.6 mmol scale with good results (Table 4, entry 6), demonstrating that the tyrosinase-mediated phenylhydrazide cleavage is also applicable on a gram scale.

The methodology was finally applied to the synthesis of longer peptides as demonstrated in Table 5. The tetra- and hexapeptides given in Table 5 were synthesized from phenylhydrazide-protected dipeptides by repetitive deprotection−fragment coupling. As an illustrative example, the synthesis of Boc-Gly-Leu-Met-Gly-Met-Gly-NHNHPh (**37**) is depicted in Scheme 4.

The deprotection of the peptides shown in Table 5 proceeded smoothly except for the Fmoc-protected hexapeptide (**39**, Table 5, entry 3). Again, no attack at the peptide bonds, no cleavage of the N-terminal protecting group, and no oxidation of methionine was observed. These examples underscore that the enzymatic phenylhydrazide cleavage offers a new, orthogonal protecting group option in repetitive, multistep synthesis sequences of oligopeptides. Its application should be of considerable interest especially in the synthesis of sensitive peptide conjugates, where the common protecting groups can be applied only with difficulty.

Mechanistic Considerations. To determine whether the phenylhydrazide cleavage occurs through an *o*-hydroxylated intermediate[29] or via a different process,

(28) Nevertheless, benzoic acid phenylhydrazide is already slowly oxidized by O_2 in neutral phosphate buffer without tyrosinase and more rapidly after tyrosinase addition. Toluenesulfonic acid phenylhydrazide decomposes spontaneously in buffer without further oxygen addition.

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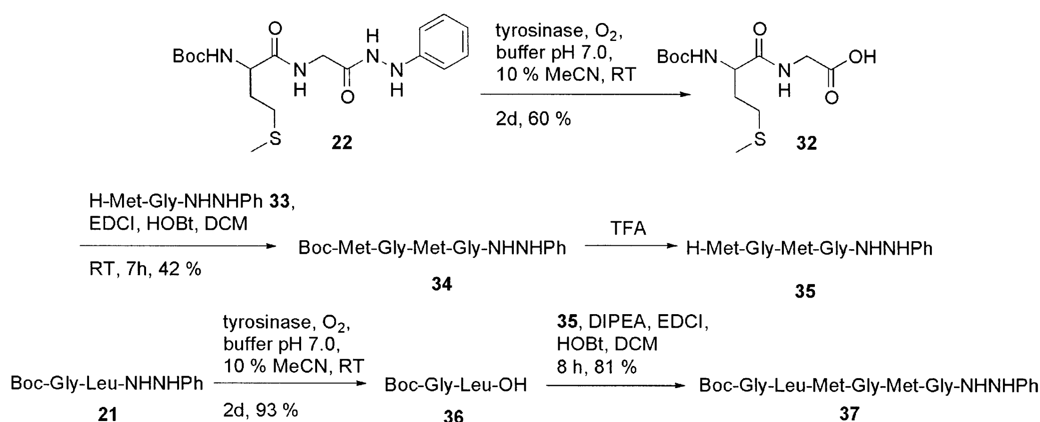
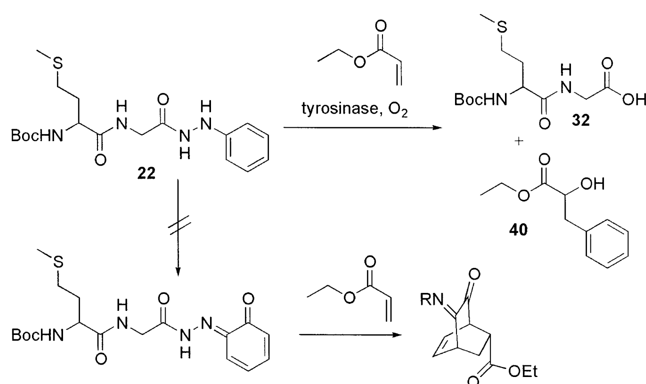
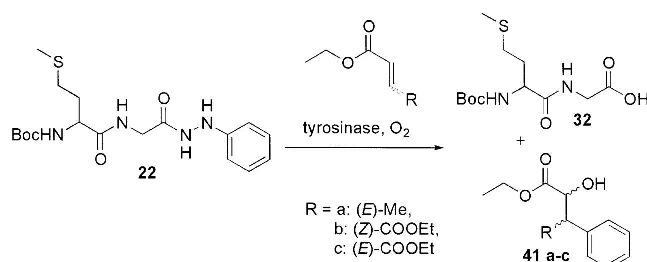
SCHEME 4. Synthesis of Boc-Gly-Leu-Met-Gly-Met-Gly-NHNHPh (37)



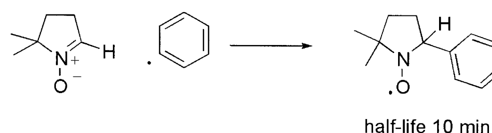
SCHEME 5. Phenylhydrazide Deprotection in the Presence of Ethyl Acrylate



SCHEME 6. Phenylhydrazide Deprotection in the Presence of Ethyl Acrylate Derivatives



SCHEME 7. Trapping of Phenyl Radicals with DMPO



we tried to trap possible intermediates. In these experiments, the deprotection of Boc-Met-Gly-NHNHPh (**22**) was performed in the presence of ethyl acrylate as illustrated in Scheme 5.

As expected, in these experiments indeed one new adduct was formed, but surprisingly this did not result from a Diels–Alder-reaction.[24] Instead, surprisingly the addition of a phenyl and a hydroxyl group to the double bond yielding **40** as the only new product was recorded. Products formed via the expected tyrosinase mechanism (cresolase- and catecholase-activity, i.e., adducts derived from phenolic or *o*-quinone fragments) were not identified. Control experiments were performed with ethyl acrylate and the phenylhydrazide alone in the absence of biocatalyst and with ethyl acrylate and tyrosinase, but without the substrate. In both cases, no reaction took place.

Likewise, crotonic acid ethyl ester and fumaric and maleic acid diethyl esters were subjected to this reaction (Scheme 6).

Interestingly, the product **41c** formed from diethyl fumarate exhibited the same 4:5 diastereomeric product distribution as did the compound **41b** formed from diethyl maleate. Based on these findings, we considered it very likely that either the enzymatic reaction itself takes place following a free-radical mechanism or that at least the hydrolysis of the putative acyldiazene (see Scheme 1) formed by enzymatic oxidation generates a phenyl radical.

This possibility was substantiated by means of electron spin resonance (ESR) spectroscopy experiments. Since we expected the actual concentration of the free radicals in the reaction mixture to be too small to be directly observable, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) was used as a spin trap. This commonly used spin trap has been reported to trap phenyl radicals under physiological conditions, thus forming radicals of a half-life of 10 min (Scheme 7).[30]

The trapping experiments were performed in 300 μL of phosphate buffer with 2350 U of the enzyme. As the substrate 35 mM H_2N-Met-Gly-NHNHPh·TFA **33** was used, and 90 mM DMPO was employed. The N-terminal protecting group had to be removed first to achieve sufficient solubility of the substrate in the buffer. After the components were mixed, oxygen was bubbled through the solution for 7 min for sufficient saturation. The experiments clearly verified the generation of free radicals during the enzymatic reaction. The observed ESR-spectrum is in excellent accordance with the spectrum reported in the literature for the phenyl-DMPO radical,[30] showing a 1:1:1 triplet due to coupling with the nitrogen atom and a doublet due to coupling with the proton (Figure 1).

This radical could be observed in total for more than 20 h. The radical signal built up within 1 h, then

(30) Reszka K. J.; Chignell, C. F. *Chemico-Biological Interact.* **1995**, *96*, 223–234.

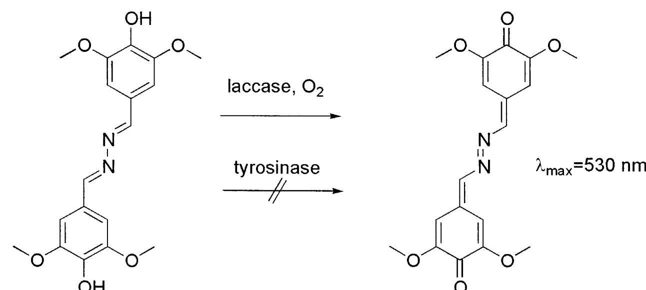
FIGURE 1. ESR spectrum of the reaction mixture (2350 U tyrosinase, 33 mM Met-Gly-NHNHPh*TFA **33**, 90 mM DMPO, O_2) after 60 min; $a_N = 15.83$ G and $a_H = 24.55$ G, $g = 2.0054$.

remained in a steady state, and finally faded with a half-time of about 4 h. This half-time is of course dependent on the rate of radical formation that is determined by the amount of enzyme, the concentrations of substrate and O_2 and by the lifetime of the radical. In the chosen experimental setup, it is not possible to determine the real lifetime of the radical. The fading of the signal reflects a decline in free-radical formation due to consumption of either substrate or the cofactor O_2, but possibly also due to inhibition of the enzyme. An otherwise identical blank sample without tyrosinase did not show any free-radical formation during 10 h.

These ESR experiments cannot resolve whether the enzymatic reaction itself generates a free-radical intermediate or whether radical formation occurs during nonenzymatic hydrolysis of the acyl diazene.[31] Still, considering the fact that the regular reactions catalyzed by the enzyme are an *o*-hydroxylation and a catechol oxidation and that in our experiments phenolic or catecholic intermediates could never be identified, we consider it likely that the enzymatic phenylhydrazide cleavage follows a mechanism differing from these established modes of action.

Such a different mechanism was also postulated by Pandey et al. for the oxidative coupling of hindered phenols[32] and by Krol and Bolton when they examined the oxidation of o-substituted 4-alkylphenols by tyrosinase.[33] In the latter case, the reaction actually turned out to be catalyzed by laccase that was present as an impurity in different batches of commercial tyrosinase preparations.[34,35] To clarify whether also the deprotection of phenylhydrazides might occur by means of a potential laccase contamination, we determined the laccase activity of our enzyme preparation using the syringaldazine assay[36,37] (Scheme 8).

SCHEME 8. Oxidation of Syringaldazine by Laccase



In a first experiment, the laccase activity of the enzyme preparation was determined for 24 h. During this period, no increase in absorbance at 530 nm was observed. To quantify the sensitivity of the assay, also mixtures of 20 U laccase (supplied from Sigma) together with 0, 1, 4, and 20 mass equiv of tyrosinase were examined. In these experiments, we observed that the syringaldazine oxidation was slowed according to the amount of tyrosinase present, indicating a competition between tyrosinase and laccase for binding of syringaldazine.

On the basis of these experiments, we propose that at least the second, hydrolytic step (Scheme 1) of the phenylhydrazide cleavage occurs through a free-radical mechanism and that the oxidative step does not involve o-hydroxylation.

Conclusion

We have demonstrated the versatility of amino acid phenylhydrazides as enzyme-labile C-terminal protecting groups in the synthesis of peptides and peptide conjugates. The protecting group is removed by an enzymatic oxidation step with mushroom tyrosinase in good to excellent yield and with very high chemo- and regioselectivity. Especially remarkable is the fact that methionine was not oxidized during the enzymatic phenylhydrazide removal. The biocatalyst can be isolated from common mushrooms via a simple and inexpensive procedure in large quantities and sufficient purity. The enzymatic deprotection is also applicable to the synthesis of lipopeptides, although the solubility of the substrates appears to be a limiting factor. The phenylhydrazide group is fully orthogonal to the Boc-, Z-, and Fmoc blocking functions. The use of the Fmoc group is somewhat restricted due to the poor solubility of the corresponding substrates under the conditions of the enzymatic reactions, leading to lower conversion and yield in the enzymatic cleavage steps.

Due to the fact that the enzymatic reaction does not include a nucleophilic attack on a carbonyl group as it is the case for hydrolases, the phenylhydrazide group should also be orthogonal toward other enzymatically cleavable protecting groups such as enzyme labile esters[8,38] or the PhAcOZ-urethane.[10,39] In particular, in the

(31) Additional ESR-investigations with Boc-Pro-N=N−Ph isolated after NBS-oxidation and DMPO in phosphate buffer confirm that the hydrolysis indeed occurs under formation of a phenyl radical. Interestingly, this free radical formation is slowed, but not suppressed in the presence of tyrosinase, a fact that we do not truly understand yet.

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synthesis of very acid- and base-labile peptide conjugates, one of the most demanding test cases for any protecting group strategy in respect to the mildness of reaction conditions and selectivity, the enzymatic cleavage of phenyl hydrazides should be welcome as an entirely new, fully orthogonal and very mild methodology.

Experimental Section

Tyrosinase was isolated by the $(NH_4)_2SO_4$ precipitation procedure described for preparation 1 in ref 15. Typically, the activities of these enzyme preparations lie in a range of 350–450 U/mg. The HIC-purified enzyme preparation (preparation 2) showed an activity of 1050 U/mg. CH_2Cl_2 was distilled from CaH_2 prior to use. All reactions except for the enzymatic deprotections were carried out under an atmosphere of argon. In NMR spectra, multiplicities are given as singlet (s), doublet (d), triplet (t), quadruplet (q), broad (b). Coupling constants are given in Hz. ^{13}C-H substitution was determined with a DEPT-135 pulse sequence, differentiating signals of methyl and methine carbons pointing "up" (+) from methylene carbons pointing "down" (−) and quaternary carbons that are missing (o). GC–MS spectra were recorded using a Macherey&Nagel Optima 1 (0.2 mm × 25 m) capillary column and the following temperature program: injector: 150 °C, oven: 100 °C (0 min), 100 °C (2 min), 300 °C (12 min), 300 °C (20 min). HPLC–ESI–MS spectra were recorded using Macherey&Nagel analytical Nucleosil C4 or C18HD columns and the following gradient program: flow: 1 mL/min, solvent A: 0.1% HCOOH in H_2O, solvent B: 0.1% HCOOH in MeCN, A/B 80/20 (0 min), 50/50 (20 min), 10/90 (30 min), 0/100 (40 min), 80/20 (41 min), 80/20 (45 min). HPLC purities were determined by integration of the 210 nm trace. Melting points are reported uncorrected. The ESR spectra were recorded on a Varian E-109 spectrometer at 8.980788 GHz using the following instrumental conditions: modulation frequency 100 kHz, modulation amplitude 1 G, power 5 mW, sweep time 15 min, time constant 1 s. The *g* value was determined using 2,2-di(4-*tert*-octylphenyl)-1-picrylhydrazyl free radical (DPPH) as a reference ($g = 2.0037$) and a frequency counter (Hewlett-Packard, 5246 L) with a frequency converter (HP 5255 A).

General Procedures. Tyrosinase activity assay: One unit is defined as the amount of enzyme that produces a $\Delta_{280\ nm}$ of 0.001 per minute in a phosphate-buffered 0.3 mM tyrosine solution (pH 6.5).

Laccase Activity Assay. One unit produces a $\Delta_{530\ nm}$ of 0.001 per minute at pH 6.5 in a solution containing 0.02 mM syringaldazine. With this assay, no formation of the adduct was observed in the tyrosinase preparation within 24 h.

Synthesis of N-Terminally Protected Amino Acid Phenylhydrazides. Phenylhydrazine (1.1 equiv, for Fmoc-protected amino acids 0.9 equiv) was added at 0 °C to a solution of the amino acid, 1.2 equiv of carbodiimide, and 1.4 equiv of HOBt in CH_2Cl_2 (5 mL/mmol). After 1 h, the reaction mixture was allowed to warm to room temperature and stirred until completion of the reaction (TLC monitoring). The reaction mixture was washed three times with each 1 N HCl, saturated $NaHCO_3$, and brine, the organic layer was dried over $MgSO_4$, and the solvent was removed in vacuo. The crude product was purified by recrystallization or flash column chromatography.

C-Terminal Elongation of Amino Acid and Dipeptide Phenylhydrazides. The amine (1.0 equiv, in 3–5 mL/mmol CH_2Cl_2, in the case of TFA salts with 1.5 equiv of DIPEA) was added at 0 °C to a solution of the free amino acid, 1.2 equiv of carbodiimide, and 1.4 equiv of HOBt in CH_2Cl_2 (5–10 mL/mmol). After 1 h, the reaction mixture was allowed to warm to room temperature and stirred until completion of the reaction (TLC-monitoring). The reaction mixture was washed three times with each 1 N HCl, saturated $NaHCO_3$, and brine, the organic layer was dried over $MgSO_4$, and the solvent was removed in vacuo. The crude product was purified by flash column chromatography.

Enzymatic Deblocking of Amino Acid Phenylhydrazides. The amino acid phenylhydrazide was dissolved in phosphate buffer (pH 7.0, 50–100 mM, 50–200 mL/mmol) with the required amount of cosolvent (Tables 3–5). After addition of tyrosinase, oxygen was bubbled through the solution until the starting material disappeared (TLC control). **Workup A.** The cosolvent was removed under reduced pressure, and then the remaining solution was lyophilized. The remaining solid was sonicated for 5 min in 20 mL of MeOH and filtered through Celite, and the solvent was removed by evaporation in vacuo. The crude product was purified by flash column chromatography. **Workup B.** The reaction mixture was extracted with EtOAc to remove unreacted starting material, and then the pH was adjusted to 2–3 by addition of 1 N HCl. After extraction with EtOAc, the organic layer was concentrated in vacuo and the crude residue was purified by flash column chromatography.

Synthesis of *N,N'*-Bis-*tert*-butyloxycarbonyl-L-cystine Bis-phenylhydrazide 7. As described in the general procedure, 4.2 mmol (1.85 g) of Boc-L-cystine **10**, 9.24 mmol (2.2 equiv, 1.34 g) of $PhNHNH_2$·HCl, 9.24 mmol (2.2 equiv, 1.58 mL) of DIPEA, 10.08 mmol (2.4 equiv, 1.92 g) of EDCI, and 11.76 mmol (2.8 equiv, 1.80 g) of HOBt were used: yield 3.87 mmol (2.40 g, 92%); off-white solid; mp 150–160 °C dec; ^{1}H NMR ($CDCl_3$, 400 MHz) 1.43 (s, 18H), 2.99 (dd, $J = 10.2$, 14.5 Hz, 2H), 3.11 (dd, $J = 4.3$, 14.5 Hz, 2H), 5.08 (bs, 2H) 5.6 (bs, 2H) 6.85–6.93 (m, 6H), 7.20–7.26 (m, 4H), 9.6 (bs, 2H); ^{13}C NMR (100 MHz) 28.3 (+, 3C), 46.8 (−), 53.5 (+), 81.0 (o), 113.8 (+, 2C), 121.2 (+), 129.2 (+, 2C), 148.1 (o), 156.2 (o), 170.3 (o); HPLC–ESI–MS (C4) $t_R = 25.78$ min, purity 93%, M = 643.2 $[M + Na]^+$, 1263.0 $[2M + Na]^+$; M calcd for $C_{28}H_{39}N_6O_6S_2Na$ 642.2270, found 642.2289.

Synthesis of *N,N'*-(Bis-*tert*-butyloxycarbonylglycyl)-L-cystine Bis-phenylhydrazide 11. To a solution of 2.0 mmol of **7** (1.24 g) in 20 mL of CH_2Cl_2 was added 4 mL of TFA under ice-cooling. After 1 h, the solvent was distilled off in vacuo under repeated addition of 10 mL of toluene. To the residue in 20 mL of CH_2Cl_2 were added a solution of 4.2 mmol Boc-Gly-OH (736 mg, 1.05 equiv), 4.5 mmol of EDCI (863 mg, 1.15 equiv), 5.0 mmol of HOBt (766 mg, 1.25 equiv), and 4.0 mmol of DIPEA (0.68 mL, 1.0 equiv) in 10 mL of CH_2Cl_2 at 0 °C. After 1 h at this temperature, the mixture was stirred at rt for 4 h and then washed three times with each 1 N HCl, saturated $NaHCO_3$, and brine. The organic layer was dried over $MgSO_4$, and the solvent was removed in vacuo. The crude product (1.21 g) was purified by flash chromatography (SiO_2, 5% MeOH in DCM) yielding 898 mg of **11** (1.22 mmol, 61%) as an off-white solid: mp 117–119 °C dec; ^{1}H NMR ($CDCl_3$, 400 MHz) 1.42 (s, 18H), 3.02 (dd, $J = 8.0$, 14.1 Hz, 2H), 3.11 (dd, $J = 5.8$, 14.1 Hz, 2H), 3.67–3.82 (m, 4H) 4.88–5.02 (m, 2H), 6.74–6.85 (m, 6H), 7.11–7.19 (m, 4H); ^{13}C NMR(100 MHz) 28.4 (+, 3C), 44.2 (−), 51.6 (−), 59.3(+), 79.5 (o), 113.7 (+, 2C), 121.2 (+), 129.3 (+, 2C), 141.6(o), 148.1 (o), 167.8 (o), 172.2 (o); HPLC–ESI–MS (C4) $t_R = 23.09$ min, purity 88%; M = 757.2 $[M + Na]^+$, 735.0 $[M + H]^+$, 679.0 $[M - tBu]^+$, 635.1 $[M - Boc]^+$, 579.0 $[M - Boc - tBu]^+$, 535.1 $[M - 2Boc]^+$, M calcd for $C_{32}H_{46}N_8O_8S_2$ 734.2880, found 734.2884.

Synthesis of *S*-Palmitoyl-*N*-*tert*-butyloxycarbonylglycyl-L-cysteine Phenylhydrazide 12. To a solution of 0.25 mmol of **11** (184 mg) in 10 mL of CH_2Cl_2 were added 1.25 mmol of dithiothreitol (193 mg, 5 equiv) and 0.525 mmol of NEt_3 (73 μL, 2.1 equiv). The solution was stirred for 1 h, extracted three times with 1 N HCl, and dried over $MgSO_4$. At 0 °C, 0.5 mmol of palmitoyl chloride (0.151 mL, 1.0 equiv) and 0.5 mmol NEt_3 (0.070 mL, 1.0 equiv) were added. After 1 h, the solvents were removed in vacuo and the crude product was purified by flash chromatography (SiO_2, 5% MeOH in CH_2Cl_2) yielding 216 mg

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(0.36 mmol, 71%) of **12** as a yellowish oil: ^{1}H NMR ($CDCl_3$, 400 MHz) 0.88 (t, J = 7.0 Hz, 3H), 1.2−1.3 (m, 24H), 1.42 (s, 9H), 1.62−1.69 (m, 2H), 2.76 (t, J = 7.6 Hz, 2 H), 3.33−3.36 (m, 2H), 3.75−3.79 (m, 2H) 4.65−4.72 (m, 2H), 5.13 (bs, 1H), 6.02 (bd, J = 4.3 Hz, 1H), 6.80−6.91 (m, 2H), 7.04−7.07 (m, 1H), 7.19−7.24 (m, 2H), 8.63 (bs, 1H); ^{13}C NMR (100 MHz) 14.0 (+), 22.6 (−), 25.6 (−), 28.2 (+, 3C), 28.9−29.6 (all -, 10C), 30.0, 31.9, 44.0, 45.6 (all −), 55.0(+), 80.9 (o), 113.7 (+, 2C), 121.2 (+), 129.1 (+, 2C), 141.6, 148.1, 169.5, 170.3, 201.4 (all o); HPLC−ESI−MS (C4) t_R = 32.35 min, purity 86%; M = 629.3 $[M + Na]^+$, 573.3 $[M + Na - tBu]^+$, M calcd for $C_{32}H_{54}N_4O_5SNa$ 629.3713, found 629.3693.

Enzymatic Deblocking of *S*-Palmitoyl-*N*-*tert*-butyloxycarbonylglycyl-L-cysteine Phenylhydrazide 12. The deprotection of 0.116 mmol of **12** (70 mg) was carried out in 30 mL of phosphate buffer and 4 mL of MeCN with 30 mg of tyrosinase under bubbling of oxygen for 7 d. Workup A yielded 45 mg (0.087 mmol, 75%) of Boc-Gly-Cys(Pal)-OH as a yellowish solid and 17 mg (0.028 mmol, 24%) of **12**: mp 137−139 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) 0.81 (m, 3H), 1.1−1.3 (m, 24H), 1.37 (s, 9H), 1.48−1.56 (m, 2H), 2.21 (t, J = 7.6 Hz, 2H), 3.30−3.39 (m, 2H), 3.58−3.80 (m, 2H), 4.20−4.36 (m, 1H); HPLC−ESI−MS (C4) t_R = 29.92 min, purity 66%, M = 515.3 $[M - H]^-$, 1031.4 $[2M - H]^-$, M calcd for $C_{26}H_{48}N_2O_6SNa$ 539.3131, found 539.3108.

Enzymatic Deblocking of Boc-Met-Gly-NHNHPh 22. Deprotection of 1.66 mmol **22** (657 mg) is performed in a mixture of 250 mL of phosphate buffer (100 mM, pH 7.0) and 30 mL of MeCN with 290 mg of tyrosinase and with oxygen bubbling. After 3 days, workup procedure B yielded 305 mg of **32** (1.00 mmol, 60%) and 160 mg of **22** (0.40 mmol, 24%): ^{1}H NMR ($CDCl_3$ + MeOD, 400 MHz) 1.38 (s, 9H), 1.80−1.85 (m, 1H), 1.95−2.04 (m, 1H), 2.03 (s, 3H) 2.45−2.53 (m, 2H), 3.83−3.98 (m, 2H), 4.03−4.12 (m, 1H); ^{13}C NMR (100 MHz) 14.78 (+), 27.9 (+, 3C), 29.7, 31.7, 40.7 (all −), 53.1 (+), 80.0 (o), 155.8, 171.1, 172.5 (all o); HPLC−ESI−MS (C4) t_R = 9.25 min, purity 87%; M = 305.0 $[M - H]^-$, 611.0 $[2M - H]^-$ 231.1, M calcd for $C_{12}H_{23}N_2O_5S$ 307.1327, found 307.1339.

Synthesis of Boc-Met-Gly-Met-Gly-NHNHPh 34. A solution of 1.06 mmol of **22** (420 mg) in 10 mL of CH_2Cl_2 was treated at 0 °C with 2 mL of TFA for 1 h. The solvent was evaporated, and the crude product was directly employed in the subsequent coupling with 0.98 mmol of **32** (300 mg) under standard conditions as described above. Flash chromatography (SiO_2, 3% MeOH in DCM) yielded 243 mg (0.42 mmol, 42%) of a yellowish solid: mp 136−138 °C dec; ^{1}H NMR ($CDCl_3$ + MeOD, 400 MHz) 1.41 (s, 9H), 1.75−1.85 (m, 1H), 1.90−2.04 (m, 2H), 2.05 (s, 3H), 2.06 (s, 3H), 2.11−2.24 (m, 1H), 2.41−2.58 (m, 4H), 3.70−3.77 (m, 1H), 3.86−3.95 (m, 1H), 3.95−4.07 (m, 2H), 4.12 (dd, J = 5.5, 8.4 Hz, 1H), 4.46 (dd, J = 5.4, 8.7 Hz, 1H), 6.72−6.88 (m, 3H), 7.14−7.20 (m, 2H); ^{13}C NMR (100 MHz) 14.79, 14.83 (both +), 27.9 (+, 3C), 29.8, 29.9, 30.1, 30.9, 41.6, 42.7 (all −), 52.8, 53.8 (both +), 80.2 (o), 113.1 (+, 2C), 120.5 (+), 128.6 (+, 2C), 147.5, 156.2, 169.4, 170.3, 172.4, 173.6 (all o); HPLC−ESI−MS (C4) t_R = 15.37 min, purity 91%, M = 583.1 $[M - H]^-$, M Calcd for $C_{25}H_{41}N_6O_6S_2$ 585.2529, found 585.2529.

Enzymatic Deblocking of Boc-Met-Gly-Met-Gly-NHNHPh 34. Deprotection of 0.125 mmol of **34** (73 mg) was carried out in a mixture of 30 mL of phosphate buffer and 3 mL of MeCN with 40 mg of tyrosinase under oxygen bubbling. After 24 h, workup B yielded 50 mg of Boc-Met-Gly-Met-Gly-OH (0.101 mmol, 81%) as a brownish oil and 7 mg of **34** (0.012 mmol, 10%): ^{1}H NMR ($CDCl_3$ + MeOD, 400 MHz) 1.39 (s, 9H), 1.79−1.95 (m, 2H), 1.95−2.02 (m, 1H), 2.04 (s, 3H), 2.05 (s, 3H), 2.06−2.14 (m, 1H), 2.44−2.56 (m, 4H), 3.73−3.80 (m, 1H), 3.89−3.93 (m, 2H), 3.95−4.03 (m, 1H), 4.20 (dd, J = 5.2, 8.3 Hz, 1H), 4.57 (dd, J = 5.7, 8.2 Hz, 1H); ^{13}C NMR (100 MHz) 15.00, 15.06 (both +), 28.1 (+, 3C), 29.9, 30.0, 30.9, 31.5, 40.9, 42.7 (all −), 52.1, 53.6 (both +), 80.3, 156.0, 169.6, 171.5, 171.9, 173.0 (all o); HPLC−ESI−MS (C4) t_R = 10.01 min, purity 81%, M = 493.1 $[M - H]^-$, M calcd for $C_{19}H_{35}N_4O_7S_2$ 495.1947, found 495.1957.

Enzymatic Deblocking of Boc-Gly-Leu-NHNHPh 21. Deprotection of 0.53 mmol of **21** (200 mg) is carried out in a mixture of 210 mL of phosphate buffer (100 mM, pH 7.0) and 9 mL of MeCN with 70 mg of tyrosinase under oxygen bubbling. Workup B after 2 days yields 142 mg of **36** (0.49 mmol, 93%) as colorless crystals and 10 mg of **21** (0.027 mmol, 5%): mp 137−140 °C; ^{1}H NMR ($CDCl_3$ + MeOD, 400 MHz) 0.88−0.92 (m, 6H) 1.41 (s, 9H), 1.52−1.68 (m, 3H), 3.66−3.82 (m, 2H), 4.46−4.52 (m, 1H); ^{13}C NMR (100 MHz) 21.3, 22.5, 24.6 (all +), 27.9 (+, 3C), 40.7, 43.4 (both −), 50.4 (+), 80.0 (o), 156.3, 170.0, 174.6 (all o); HPLC−ESI−MS (C4) t_R = 9.85 min, purity 83%, M = 287.1 $[M - H]^-$, 575.1 $[2M - H]^-$, M calcd for $C_{13}H_{24}N_2O_5Na$ 311.1583, found 311.1591.

Synthesis of Boc-Gly-Leu-Met-Gly-Met-Gly-NHNHPh 37. A solution of 0.205 mmol of **34** (120 mg) in 10 mL of CH_2Cl_2 was stirred with 2 mL of TFA at 0 °C for 1 h. The solvent was evaporated, and the crude residue was directly employed in the subsequent coupling with 0.25 mmol of **36** (95 mg) under standard conditions as described above. Flash chromatography (SiO_2, 3% MeOH in DCM) yielded 125 mg (0.165 mmol, 81%) of a yellowish foam: mp 185−187 °C; ^{1}H NMR ($CDCl_3$ + MeOD, 400 MHz) 0.80−0.91 (m, 6H), 1.41 (s, 9H), 1.44−1.61 (m, 3H), 1.95−2.15 (m, 10H), 2.46−2.58 (m, 4H), 3.58−3.89 (m, 6H), 4.13−4.26 (m, 2H), 4.48−4.54 (m, 1H), 6.76−6.83 (m, 3H), 7.14−7.18 (m, 2H); HPLC−ESI−MS (C4) t_R = 18.57 min, purity 81%, M = 755.1 $[M + H]^+$, M = 777.3 $[M + Na]^+$, M = 655.1 $[M - Boc + H]^+$, M calcd for $C_{33}H_{54}N_8O_8S_2Na$ 777.3404, found 777.3404.

Enzymatic Deblocking of Boc-Gly-Leu-Met-Gly-Met-Gly-NHNHPh 37. Deprotection of 0.106 mmol **37** (80 mg) was carried out in a mixture of 210 mL of phosphate buffer (100 mM, pH 7.0) and 9 mL of MeCN with 70 mg of tyrosinase under oxygen addition. After 15 h, workup B yielded 40 mg of Boc-Gly-Leu-Met-Gly-Met-Gly-OH (0.060 mmol, 57%) and 18 mg of **37** (0.024 mmol, 22%): ^{1}H NMR (D_2O, 400 MHz) 0.72−0.83 (m, 6H) 1.30 (s, 9H), 1.44−1.56 (m, 3H), 1.85−2.09 (m, 6H), 2.13−2.26 (m, 1H), 2.37−2.54 (m, 2H), 2.59 (bs, 3H), 2.76−2.88 (m, 2H), 3.62−3.74 (m, 2H), 3.78−3.95 (m, 4H), 4.21−4.27 (m, 1H), 4.31−4.46 (m, 2H); HPLC−ESI−MS (C4) t_R = 14.08 min, purity 55%, M = 663.1 $[M - H]^-$.

Trapping Experiments in the Presence of Ethyl Acrylate: Formation of 40. A mixture of 0.253 mmol of **22** (100 mg) was treated with 50 mg of tyrosinase in 50 mL of buffer, 3 mL of MeCN, and 1.5 mmol of ethyl acrylate (150 mg, 6 equiv) with oxygen bubbling for 12 h. The pH was adjusted to 8−9 by addition of $NaHCO_3$, and the mixture was extracted three times with 50 mL of EtOAc. The organic layer was dried over $MgSO_4$ and concentrated in vacuo, and the crude residue was purified by flash chromatography (SiO_2, DCM/EtOAc 95:5) yielding 19 mg of **40** (0.098 mmol, 39%). The pH of the aqueous layer was adjusted to pH 2 and the mixture is extracted with EtOAc to yield 45 mg **32** (0.147 mmol, 58%): ^{1}H NMR ($CDCl_3$, 400 MHz) 1.26 (t, J = 7.1 Hz, 3H), 2.78 (bd, J = 6 Hz, 1H, exchangeable), 2.95 (dd, J = 6.9, 13.9 Hz, 1H), 3.10 (dd, J = 4.5, 13.9 Hz, 1H), 4.20 (q, J = 7.1 Hz, 2H), 4.41 (bdd, J = 4.6, 6.7 Hz, 1H), 7.18−7.30 (m, 5H); ^{13}C NMR (100 MHz) 14.39 (+), 40.80 (−), 61.93 (−), 71.46 (+), 127.1 (+), 128.6 (+, 2C), 129.8 (+, 2C), 136.6 (o), 174.4 (o); GC−MS t_R = 7.331 min, purity >99%, M = 194 $[M]^+$, 176 $[M - H_2O]^+$, 91, 65, M calcd for $C_{11}H_{14}O_3$ 194.0943, found 194.0869.

ESR Experiments. Substrate solution: 100 mM **33** in 100 mM phosphate buffer pH 7.0. Spin trap solution: 200 mM 5,5-dimethylpyrroline *N*-oxide (DMPO) in phosphate buffer. Immediately before the measurements, 100 μL of buffer, substrate, and DMPO solutions were added to 5.56 mg of tyrosinase, and the solution was treated with O_2 for 7 min. For the blank sample, 100 μL of buffer, substrate, and DMPO solution were treated with O_2 for 7 min without tyrosinase.

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Supporting Information Available: Characterization of compounds **1**−**6**, **8**, **9**, **13**−**16**, **18**, **19**, **21**−**31**, **38**, **39**, **41a**, and **41b**/**c**; enzymatic deblocking of **1**−**5**, **9**, **13**−**20**, **23**−**31**, **38**, and **39**. This material is available free of charge via the Internet at http://pubs.acs.org.

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