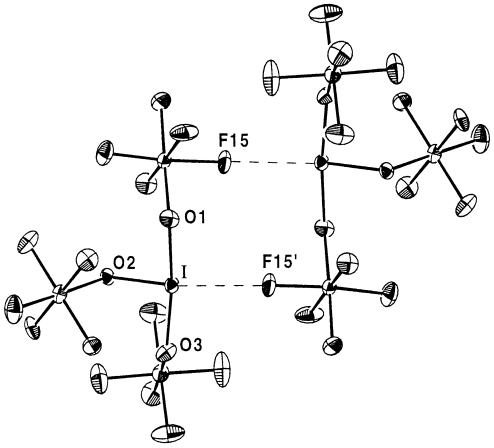

Figure 2. Crystalline structure of $I(OTeF_5)_3$ (ORTEP representation). Bond lengths [pm] and angles [°]: I-O1 208.3(5), I-O2 193.5(5), I-O3 203.3(6), I···F15 283.3(5), Te-O 183.9(5)–190.8(5), Te-F 180.8(5)–184.4(5) (F15); O1-I-O2 86.8(2), O1-I-O3 170.6(2), O2-I-O3 88.2(2) O_2-I···F15′ 171.2(2).

until the IF_3 dissolved largely or totally on shaking and the HF solution was a yellow-brown color. Warming to $-30\,^{\circ}C$ and slow cooling to $-78\,^{\circ}C$ yielded IF_3 as light-yellow platelets. With an especially designed apparatus[16] a suitable crystal was mounted on a Bruker SMART CCD 1000 TM diffractometer. Crystal structure analysis: orthorhombic, space group *Pcmn*, $a = 465.0(1)$, $b = 665.5(1)$, $c = 875.5(1)$ pm, $V = 270.9 \times 10^6$ pm^3, $T = -135\,^{\circ}C$, $Z = 4$, $Mo_{K\alpha}$ radiation, graphite monochromator, scan width of $0-3\omega$, illumination time 10 s per frame, 3279 measured, 465 independent reflections, 23 parameters, $R(F \geq 4\sigma(F)) = 0.031$, $wR_2 = 0.058$. After semi-empirical absorption correction (SADABS) the structure was solved and refined by the SHELX programs.[17]

$I(OTeF_5)_3$:[18] In a quartz vessel $CFCl_3$ (20 mL), IF_5 (1.65 g, 7.5 mmol) and $B(OTeF_5)_3$ (9.13 g, 12.5 mmol) are condensed on 635 mg (2.5 mmol) iodine at $-196\,^{\circ}C$. After the mixture had been stirred at room temperature for 2 h all volatile materials were removed under vacuum. An orange-red liquid residue slowly crystallized; the yield was almost quantitative. ^{19}F NMR (n-C_6F_{14}): Ab_4 spectrum, $\delta_A = -48.21$, $\delta_B = -45.55$, $J(AB) = 175$ Hz, $J(^{125}Te, F) = 3699$ Hz; Raman spectrum (cryst., $-150\,^{\circ}C$): 807 (20), 760 (16), 750 (8), 738 (15, sh), 730 (22), 719 (57), 713 (32, sh), 700 (62), 689 (60), 666 (97), 642 (15, sh), 635 (35), 596 (42), 492 (60), 470 (100), 449 (65), 390 (12), 346 (15), 326 (32), 314 (22, sh), 298 (25), 254 (37), 235 (59), 228 (31, sh), 215 (11), 201 (12), 177 (49), 162 (22), 130 (85), 109 (16), see also ref. [18]. Crystal structure analysis: monoclinic, space group $P2_1/c$, $a = 1447.8(1)$, $b = 973.4(1)$, $c = 1027.4(1)$ pm, $\beta = 91.42(1)$, $V = 1447.4 \times 10^6$ pm^3, $T = -128\,^{\circ}C$, $Z = 4$, 14936 measured, 1485 independent reflections, $R(F \geq 4\sigma(F)) = 0.049$, $wR_2 = 0.128$.

Further details on the crystal structure investigations may be obtained from the Fachinformationszentrum Karlsruhe, 76344 Eggenstein-Leopoldshafen, Germany (fax: (+49) 7247-808-666, on quoting the depository numbers CSD-411036 and -411037).

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Synthesis of the N-Terminal *N*-Myristoylated and *S*-Palmitoylated Undetrigintapeptide of Endothelial NO-Synthase**

Rainer Machauer and Herbert Waldmann*

The correct orchestration of signal transduction from the blood stream across the endothelium to the surrounding smooth muscle cells of the blood vessels is paramount to the regulation of blood pressure. A key event in this regulation is the generation and release of NO from arginine by endothelial NO-synthase (eNOS) in response to exogeneous signals and the subsequent relaxation of the muscle cells.[1, 2] Furthermore eNOS is involved in vascular remodelling and angiogenesis,[3] and contributes to the pathogenesis of blood vessel related disorders like atheriosclerosis.[4, 5] The localization of eNOS to the plasma membrane and its concentration in the caveolae, membrane microdomains highly enriched in various signal transducing proteins is crucial to its correct biological functioning.[6]

In contrast to the other isoforms of NO-synthase identified so far, the N-terminus of eNOS is *N*-myristoylated and twice *S*-palmitoylated (see Figure 1 and **1**, Scheme 1).[7] The lipid

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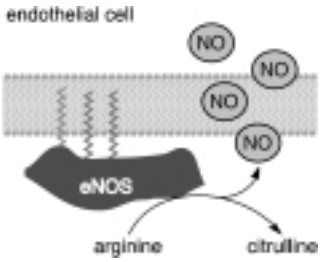
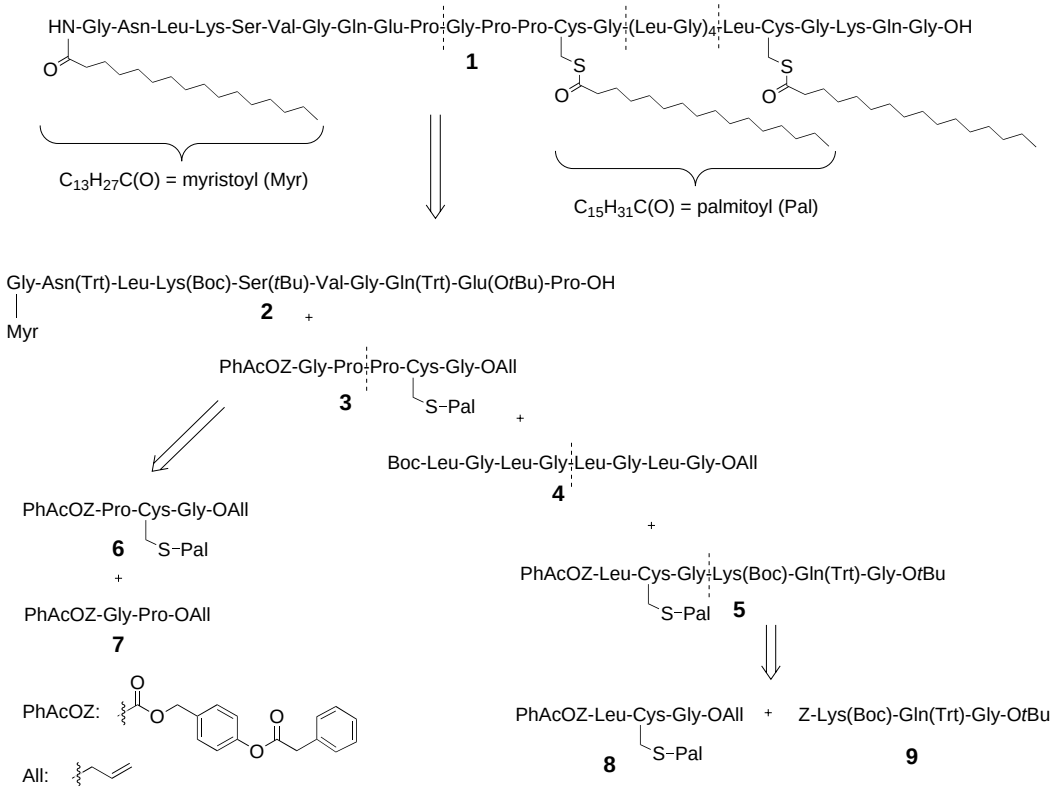


Figure 1. Membrane association and enzymatic activity of endothelial NO-synthase.

The synthesis of lipidated peptide **1** is complicated by several parameters: 1) the palmitic acid thioesters are sensitive to bases; that is, they spontaneously hydrolyze at pH > 7 and are subject to base-induced β-elimination.[9] Therefore, base-labile protecting groups can not be employed in the synthesis of **1**. 2) For the synthesis of *S*-palmitoylated peptides solid-phase methods are not available.[10] 3) Peptide **1** embodies several amino acids with side-chain functional groups that require protection during the synthesis and final deprotection under conditions mild enough to guarantee that the sensitive thioester bonds are not attacked.

In developing a plan for the synthesis of **1** we chose a combination of enzyme-labile, acid-sensitive, and noble-metal-sensitive protecting groups and aimed to combine the use of solid-phase techniques with solution-phase fragment condensations. Thus, in a retrosynthetic sense, **1** was divided into *N*-myristoylated decapeptide **2**, *S*-palmitoylated pentapeptide **3**, octapeptide **4**, and *S*-palmitoylated hexapeptide **5** (Scheme 1). For the synthesis and selective deprotection of *S*-palmitoylated building blocks the enzyme-labile *p*-phenylacetoxybenzyloxycarbonyl (PhAcOZ) urethane group[11] and the Pd^0-labile allyl ester[12] were chosen as N- and C-terminal protecting functions. The side chains of Asn, Lys, Ser, Gln, and Glu were masked with acid-labile protecting groups to be cleaved off simultaneously in the final step of the synthesis. We planned to build up the N-terminal decapeptide on the solid phase and to assemble the entire 29mer in solution by appropriate fragment condensations. The retrosynthetic cuts were placed at the C-termini of glycine and proline residues to exclude epimerization during the fragment condensations.



Scheme 1. Retrosynthetic analysis of the *N*-myristoylated and twice *S*-palmitoylated N-terminal 29mer peptide of endothelial NO-synthase. Boc = *tert*-butoxycarbonyl, Trt = trityl = triphenylmethyl, Z = benzyloxycarbonyl.

groups are required for plasma membrane localization and biological activity.[6, 8] However, the precise biological roles fulfilled by the lipidated part of the protein are subject to various hypotheses. In particular, notions have been forwarded that the lipid groups might be responsible for selective targeting of eNOS at the plasma membrane and caveolae, for example, by mediatiation of protein/protein or protein/lipid interactions, and that palmitoylation/depalmitoylation might be involved in signalling through eNOS.[5]

Lipidated peptides which embody the characteristic partial structures of their parent lipidated proteins have proven to be efficient tools for subsequent biological experiments, for example, in the case of the *Ras* proteins.[9] For the study of the chemical biology of endothelial NO-synthase, and in particular, for the study of the factors determining its localization to the plasma membrane and caveolae, we have now developed a methodology that gives access to the correctly lipidated N-terminal 29mer peptide **1** of eNOS (Scheme 1).

The base labile *S*-palmitoylated building blocks required for the fragment condensations were synthesized as shown in Scheme 2. PhAcOZ-protected amino acids **10** and **11** were condensed with cystine bis(allyl ester) **12**. After cleavage of the disulfide by treatment with dithiothreitol (DTT), the liberated mercapto groups were acylated with palmitoyl chloride to yield fully masked dipeptides **13** and **14** in high overall yields. To elongate the peptide chain in the C-terminal direction, the allyl ester protecting group was selectively

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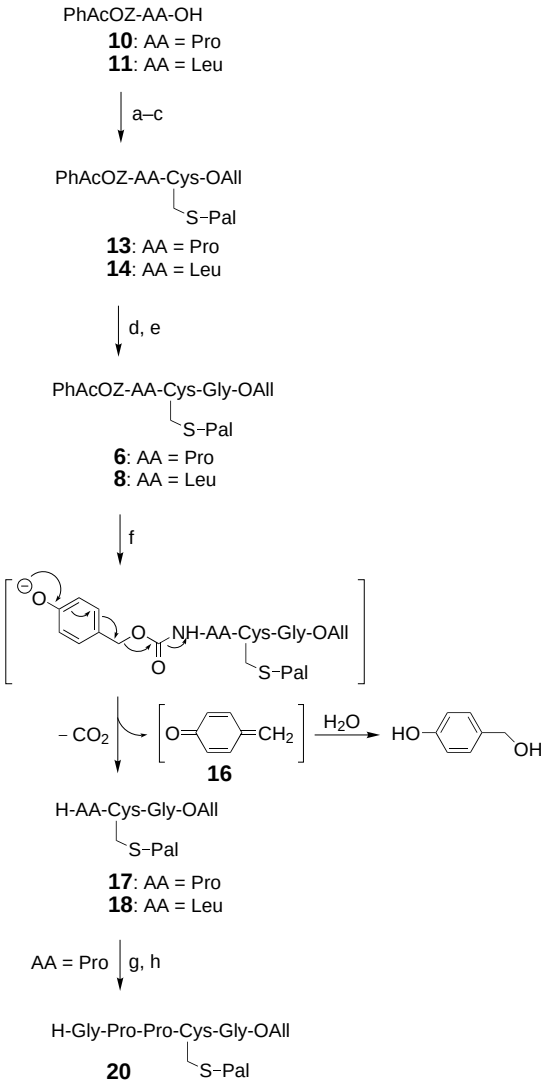


Scheme 2. Synthesis of the sensitive *S*-palmitoylated building blocks with a combination of the enzyme-labile PhAcOZ urethane and the Pd^0-sensitive allyl ester protecting groups. a) [H-Cys-OAll]$_2 \cdot 2p$TosOH **12**, HOBt, EDC, NEt_3, CH_2Cl_2, **10**: 88%, **11**: 90%; b) DTT, NEt_3, CH_2Cl_2; c) $H_3C(CH_2)_{14}COCl$, NEt_3, 0 °C, CH_2Cl_2, **10**: 72% (two steps), **11**: 85% (two steps); d) [$Pd(PPh_3)_4$], DMB, THF, **13**: 91%, **14**: 86%; e) H-Gly-OAll $\cdot p$TosOH **15**, HOBt, EDC, CH_2Cl_2, **6**: 91%, **8**: 94%; f) penicillin G acylase, pH 6.8, 0.2 M DMB or KI, dimethyl-β-cyclodextrin, 20% MeOH, **17**: 53%, **18**: 56%; g) PhAcOZ-Gly-Pro-OH **19**, HOAt, EDC, CH_2Cl_2, 82%; h) penicillin G acylase cross-linked enzyme crystals, pH 6.8, 0.1 M KI, dimethyl-β-cyclodextrin, 20% MeOH, 39%. HOBt = 1-hydroxybenzotriazole, EDC = *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride, DTT = 1,4-dithio-DL-threitol, DMB = *N*,*N*′-dimethylbarbituric acid, *p*-Tos = *p*-toluenesulfonyl, HOAt = 1-hydroxy-7-azabenzotriazole.

cleaved by means of Pd^0-catalyzed allyl transfer with *N*,*N*′-dimethylbarbituric acid (DMB) as the accepting C-nucleophile.[13] Under these conditions the extremely base-labile palmitic acid thioester and the N-terminal blocking function were not attacked at all. Coupling of the liberated carboxylic acids with glycine allyl ester **15** gave masked intermediates **6** and **8** which next had to deblocked at the N-terminus. Upon treatment of these PhAcOZ-protected compounds with immobilized penicillin G acylase in 0.2 M Na_3PO_4 buffer at pH 6.8, the phenylacetic acid ester incorporated into the urethane was saponified. Thereby a phenolate was generated which spontaneously fragmented into a quinone methide **16**, CO_2, and the desired selectively deprotected lipidated tripeptides **17** and **18**. Notably, the conditions of this enzyme-initiated blocking-group fragmentation are so mild that the base-labile palmitic acid thioester remained completely intact. In addition, the substrate specificity of the enzyme guarantees that the C-terminal allyl ester and the peptide bonds are not attacked.

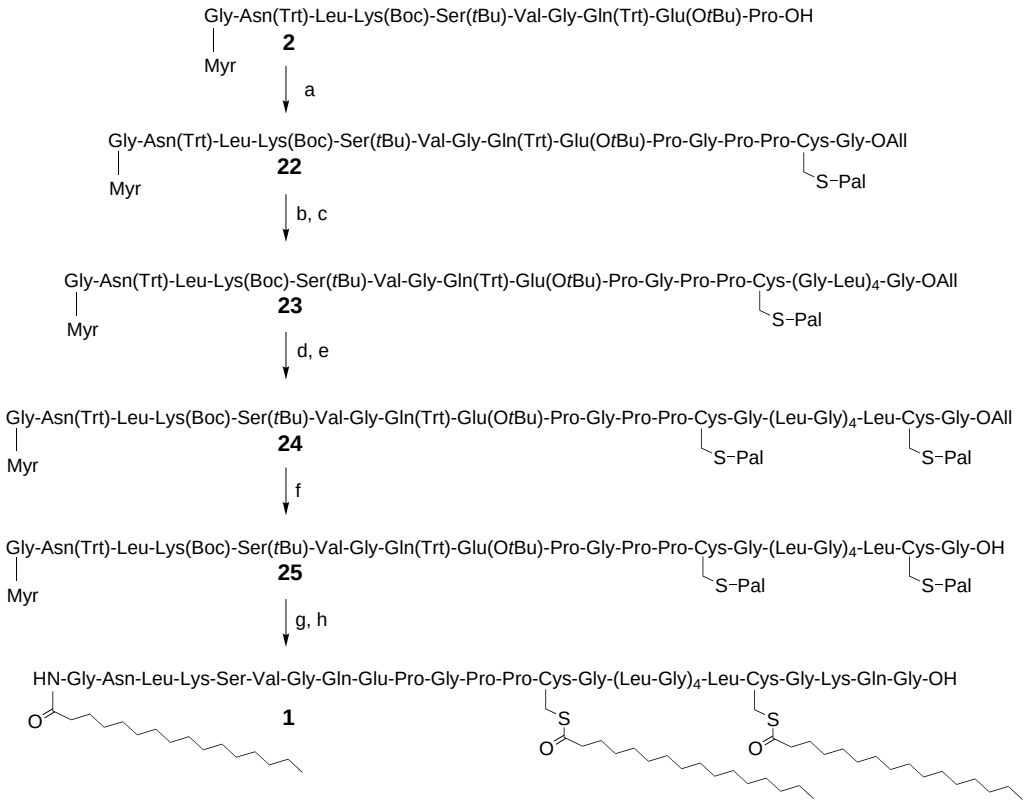
In order to achieve reproducible, preparatively useful results in these enzymatic transformations, the reactions had to be carried out in the presence of 20 vol% of methanol and 38 equivalents of dimethyl-β-cyclodextrin (for experimental details, see the supplementary material). Peptides **6** and **8** are only sparingly soluble in aqueous buffer, so that a solubilizing cosolvent is needed to render the substrates accessible to the biocatalyst. In addition, cyclodextrin most probably slips over the hydrophobic palmitoyl groups, thereby solubilizing the peptides and shielding the thioester from undesired hydrolysis. Furthermore, a nucleophile was added to trap the formed quinone methide **16** which otherwise might attack the liberated amino groups. In the case of prolyl peptide **6** the use of *N*,*N*′-dimethylbarbituric acid was best, but for leucyl peptide **8** addition of KI gave the best results. Selectively deprotected lipotripeptide **17** was then condensed with PhAcOZ-masked dipeptide **19** and the resulting lipidated pentapeptide was N-terminally deprotected by means of the enzyme-initiated fragmentation detailed above. In this case the use of cross-linked enzyme crystals (CLECs)[14] of penicillin G acylase proved to be advantageous. This preparation of the acylase is particularly stable in the presence of potentially denaturing cosolvents and gave access to the desired building block in reasonable yield. Once more the palmitic acid thioester was not attacked at all.

Leu-Gly octapeptide **4** (Scheme 1) was synthesized from Boc-Leu-Gly-OAll by selective removal of the N- and C-terminal blocking groups and condensation of the fragments to give tetrapeptide Boc-Leu-Gly-Leu-Gly-OAll (90% yield) which was then subjected to the same sequence (82% coupling yield). Selective removal of the Boc group was carried out with HCl/ether and cleavage of the allyl ester was performed with [$Pd(PPh_3)_4$]/morpholine (98–99% yields in all cases). Finally the Boc group was removed from octapeptide **4** by treatment with trifluoroacetic acid in $CHCl_3$ to yield building block $CF_3COOH{*}H(Leu\text{-}Gly)_4OAll$ **21** in 99% yield.

N-Myristoylated decapeptide **2** was synthesized on the solid phase. For N-terminal protection, the 9-fluorenylmethoxycarbonyl (Fmoc) group was employed and the side chains of the trifunctional amino acids were masked with acid-labile trityl or *tert*-butyl protecting groups. Attachment to the solid support was achieved by means of the very acid-sensitive 2-chlorotrityl linker.[15] After the entire peptide chain had been assembled, the N-terminal glycine residue was unmasked by removal of the Fmoc urethane and then the myristic acid amide was formed on the solid support by treatment with myristoyl chloride (4 equivalents) and Hünig's base (8 equivalents). Finally the desired lipidated and side-chain-protected peptide **2** was released from the polymeric carrier by treatment with acetic acid/2,2,2-trifluoroethanol/

methylene chloride (1/1/8). Peptide **2** was obtained in 98% overall yield.

With all required and selectively unmasked building blocks in hand, the assembly of the desired 29mer eNOS peptide **1** was approached (Scheme 3). To this end, decapeptide **2** was condensed with *S*-palmitoylated building block **20** to yield pentadecapeptide **22** in 91% yield. From compound **22** the C-terminal allyl ester was removed with complete selectivity (92% yield) and further chain elongation with octapeptide **21** delivered 23mer peptide **23** in high overall yield. Once more the Pd^0-catalyzed cleavage of the allyl ester proceeded efficiently and allowed for further selective elongation of the peptide chain with *S*-palmitoylated building block **18**. The selective removal of the allyl ester from the 26mer intermediate **24** was considered to be particularly challenging since this peptide embodies two base labile thioesters and several acid-labile groups. In addition, this fully masked compound is highly hydrophobic so that formation of aggregates and poor solubility, which would render the compound poorly accessible to the Pd^0-catalyst, had to be feared. However, to our great pleasure this deprotection could also be effected without any undesired side reaction, and C-terminally deprotected 26mer peptide **25** was obtained in high yield. Finally, the peptide chain was elongated with tripeptide **26** and, in the last step, all acid-labile side-chain protecting groups were cleaved by treatment with trifluoroacetic acid in the presence of ethanedithiol as a cation scavenger.



Scheme 3. Synthesis of the lipidated 29mer target peptide **1** by fragment condensation. a) H-Gly-Pro-Pro-Cys(Pal)-Gly-OAll **20**, HOOBt, EDC, $CHCl_3/CF_3CH_2OH$ (3/1), 91%; b) $[Pd(PPh_3)_4]$, DMB, DMSO, 92%; c) H-Leu-(Gly-Leu)$_3$-Gly-OAll $\cdot$ CF_3CO_2H **21**, HOAt, NEt_3, EDC, DMSO, 72%; d) $[Pd(PPh_3)_4]$, DMB, DMSO, 87%; e) H-Leu-Cys(Pal)-Gly-OAll **18**, HOAt, EDC, NMP, 86%; f) $[Pd(PPh_3)_4]$, DMB, DMSO, 69%; g) H-Lys(Boc)-Gln(Trt)-Gly-O*t*Bu **26**, HOAt, EDC, NMP, 86%; h) CF_3CO_2H/ethanedithiol/H_2O (95/2.5/2.5), 31%. HOOBt = 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine, NMP = *N*-methylpyrrolidine.

The choice of the right solvents and the work-up conditions is paramount to the success of the fragment condensations (for details, see the Supporting Information). The protected lipidated peptides **2**, **22**, **23**, **24**, and **25** are highly hydrophobic and tend to form secondary structures. Due to these properties, the peptides are only sparingly soluble in many solvents and separation of the reaction mixtures by RP-HPLC was not successful. These problems could be circumvented by running the reactions in very polar solvents and by using only a slight excess of the smaller fragments in the condensation reactions. Thus, peptide couplings were carried out with DMSO, *N*-methyl-pyrrolidinone, or $CHCl_3$/2,2,2-trifluoroethanol mixtures as solvents and all Pd^0-mediated allyl ester cleavage reactions were performed in DMSO. Use of only 1.2–1.5 equivalents of the smaller building blocks in the coupling reactions gave high coupling yields and allowed for separation of excess lipopeptide by simple washing and recrystallization due to the better solubility of the smaller monolipidated compounds.

In conclusion we have developed a highly efficient synthesis of the *N*-myristoylated and twice *S*-palmitoylated N-terminus of endothelial NO-synthase. The strategy relies on the combined use of enzyme-labile, acid-sensitive, and noble-metal-sensitive protecting groups for solution-phase synthesis of labile *S*-palmitoylated building blocks under the mildest conditions with solid-phase and fragment-condensation techniques. The results convincingly demonstrate the full capacity of the protecting group methods for the synthesis of large and multiply lipidated peptides. Together with the recently developed methods for the synthesis of entire functional proteins by a combination of organic synthesis with molecular biology,[16] they should open up new opportunities to study the chemical biology of endothelial NO-synthase in precise molecular detail, particularly the parameters determining its localization to the plasma membrane and caveolae of endothelial cells.

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4-(*N*-Methylhydrazino)-7-nitro-2,1,3-benzooxadiazole (MNBDH): A Novel Fluorogenic Peroxidase Substrate**

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Enzymes are characterized by a range of attractive features for applications in analytical chemistry. Low analyte detection limits, even in complex matrices, are realized by their high catalytic activity and substrate selectivity. In this context, peroxidases are of special interest because of the possibility of coupling H_2O_2 detection with reactions yielding H_2O_2. In addition to the type and activity of the peroxidase, the lower detection limit of the enzymatic reaction is influenced by the chromogenic or fluorogenic substrate used, as the properties of the detected reaction product play a crucial role. A variety of organic compounds are used as chromogenic substrates, for example, aromatic amines like *o*-phenylenediamine (OPD),[1] 3,3′-5,5′-tetramethylbenzidine (TMB),[2] and also 2,2′-azinobis(3-ethylbenzothiazolin)-6-sulfonate as the diammonium salt (ABTS)[3]. In case of the more sensitive fluorogenic methods the *p*-hydroxyphenylcarboxylic acids, especially *p*-hydroxyphenylacetic acid (pHPA), have found widespread application.[4]

The main disadvantage of the *p*-hydroxyphenylcarboxylic acids and other fluorogenic substrates is the difference between the optimum pH for the enzymatic reaction, which lies in a moderately acidic range,[5] and for fluorescence detection of the products, where alkaline media are required.[4] A second drawback is the short wavelengths for the excitation maxima of the fluorophores. Furthermore, oxidation of the known fluorogenic substrates yields, in most cases, a mixture of reaction products rather than one well defined fluorophore[6] and, in some cases, not even the exact structure of the fluorescent compounds obtained could be elucidated.

Hydrazine reagents are the most popular group of derivatising reagents for carbonyl compounds.[7] In this work, we describe the use of a hydrazine reagent as a fluorogenic peroxidase substrate. Surprisingly, the nonfluorescent 4-(*N*-methylhydrazino)-7-nitro-2,1,3-benzooxadiazole (MNBDH), which was recently introduced as reagent for the determination of carbonyl groups[8] and nitrite ions,[9] is oxidized by H_2O_2 in presence of peroxidase (POD) to the intensively fluorescing 4-(*N*-methylamino)-7-nitro-2,1,3-benzooxadiazole (MNBDA) [Eq. (1)]. The identity of the reaction product was verified by NMR, UV/Vis, and fluorescence spectroscopy, mass spectrometry, and HPLC. The enzymatic reaction and fluorescence detection are carried out in a mildly

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