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Synthesis of Characteristic Palmitoylated Lipopeptides from Human Y_1 Receptor by a Combination of Enzyme-Labile and Pd(0)-Sensitive Protecting Groups

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Abstract: *Base-labile palmitoylated peptides representing the characteristic lipidated region of human Y_1 receptor were synthesizea via a chemoenzymatic protecting group strategy employing both the enzyme catalyzed cleavage of the choline ester and the Pd(0)-mediated removal of the allyl ester as the key steps.*

Plasma membrane localized lipidated proteins play key roles in numerous biological signal transduction processes.[1] In particular, S-palmitoylation of cysteines has attracted a widespread interest. The highly dynamic nature of S-palmitoylation in vivo (on S-palmitoylated proteins the thioester is turned over with a half life of ca. 2 h) has led to the notion that the controlled introduction and removal of this thioester group might serve as a mechanism to trigger and terminate signal-transduction processes.[2] For the study of such signaling events peptides that embody the characteristic S-palmitoylated substructures of the parent lipoproteins may be efficient tools.[1] However, their synthesis is severely complicated by the pronounced base-lability of the palmitoyl thioesters and the tendency of acylated cysteinyl- and serinyl-peptides to undergo base-catalyzed β-elimination of the acyl groups.[3]

For the synthesis of such sensitive peptides we have introduced a set of protecting groups that can be removed under very mild conditions and without attack on the thioester bond.[1,3] However, the synthesis of lipidated peptides embodying amino acids with additional side chain functions, like carboxylic acids, calls for an even more elaborated set of orthogonally stable blocking functions. In this paper we report that this goal can be achieved by appropriate combination of enzyme-labile and Pd(0)-sensitive blocking functions namely, the choline ester[4] and the allyl ester.[5] As target peptide the characteristic lipidated partial sequence of human Y_1 receptor[6] was chosen (see **16**, Scheme 3). This G protein coupled receptor transduces signals given by neuropeptide Y, a peptide hormone with a variety of physiological functions, i. e. regulation of anxiety and hunger but also of memory and learning.[7] It is palmitoylated at cysteine 338 and the palmitoylation site is flanked by an asparagine and an aspartic acid residue[7] that requires protection in the course of the synthesis.

To develop a flexible synthesis of different palmitoylated Y_1 peptides, the target structure was assembled from three peptide units, i.e. an N-terminal, a central and a C-terminal dipeptide. The central cysteinyl peptide was built up by coupling the cystine bis(allyl ester) **1** with the Boc-phenylalanine **2**, followed by cleavage of the disulfide bond with dithiothreitol (DTT) and subsequent acylation of the liberated mercapto groups with palmitoyl chloride (Scheme 1). The fully masked dipeptide **4** obtained thereby could then be deprotected selectively at the N-terminus by treatment with trifluoroacetic acid or at the C-terminus by Pd(0)-catalyzed allyl-transfer to morpholine as the accepting nucleophile to give selectively unmasked peptides **5** and **6**. Use of the choline ester as C-terminal blocking function at this stage of the synthesis was ruled out. This protecting group would have to be generated via treatment of the corresponding palmitoylated cysteinyl 2-bromoethyl ester with trimethylamine.[4] Under these conditions base-mediated destruction of the lipopeptide is very likely to occur.

C-terminally deprotected cysteinyl dipeptide **6** was then condensed with N-terminally unmasked dipeptide **7** (for the synthesis of choline esters like **7** see ref.[4]) to give completely masked lipidated tetrapeptide **8** in high yield (Scheme 2). This intermediate embodies an enzyme-labile C-terminal blocking function and a noble-metal sensitive protecting group for the side-chain carboxylate of aspartic acid. The selective removal of the choline

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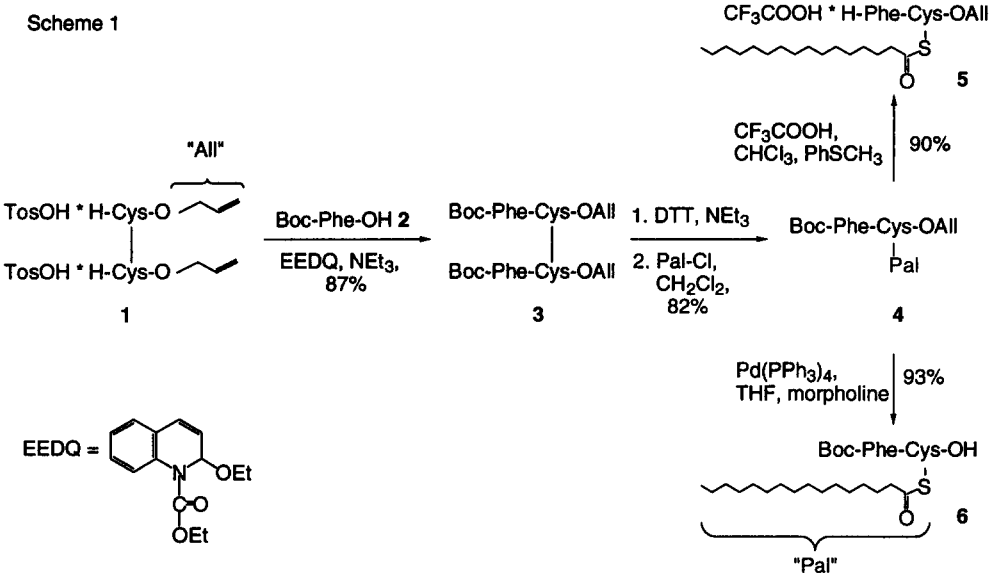
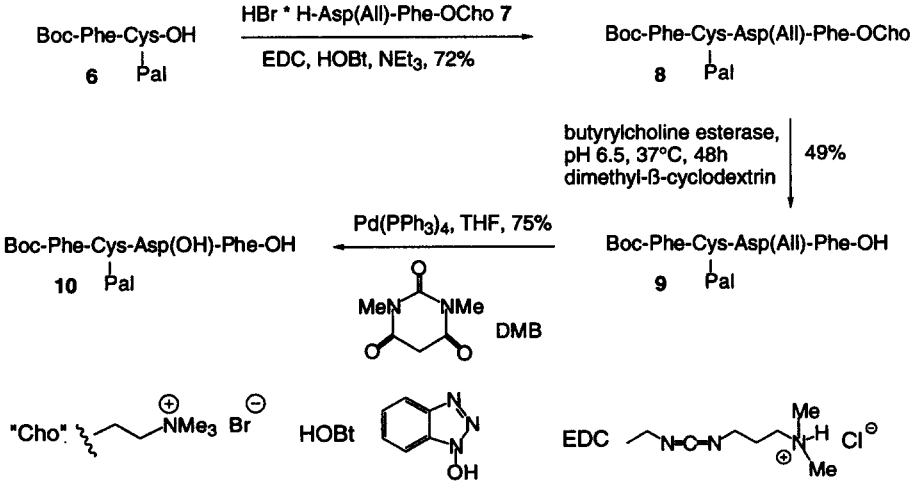
Scheme 1



ester from such a fully masked S-palmitoylated and therefore base-sensitive peptide is a considerable challenge. At pH 7 the thioester spontaneously hydrolyzes in a non-enzymatic reaction, and under these conditions the choline ester is not attacked at all.[4] Upon treatment with the enzyme butyrylcholine esterase at pH 6.5 and 37°C, however, exclusively the choline ester was saponified.[8] Neither the allyl ester nor the palmitic acid thioester were attacked and selectively unmasked tetrapeptide **9** was obtained in a smooth reaction. Thus, by means of the enzyme the chemoselectivity observed in the non-enzymatic reaction can be reversed completely. This finding demonstrates the full capacity of the enzymatic protecting group technique. In addition, under these conditions no aspartimide formation was observed. This is a notorious side reaction that usually occurs upon deprotection of aspartyl peptides under basic or acidic conditions.[9]

We note that the palmitoylated choline ester does not dissolve well in purely aqueous media. Preparatively useful yields could, however, be obtained by using additional organic cosolvents and, in particular, by adding cyclodextrins as solubility enhancers.[4] The best result was obtained in the presence of dimethyl-β-cyclodextrin.

Scheme 2

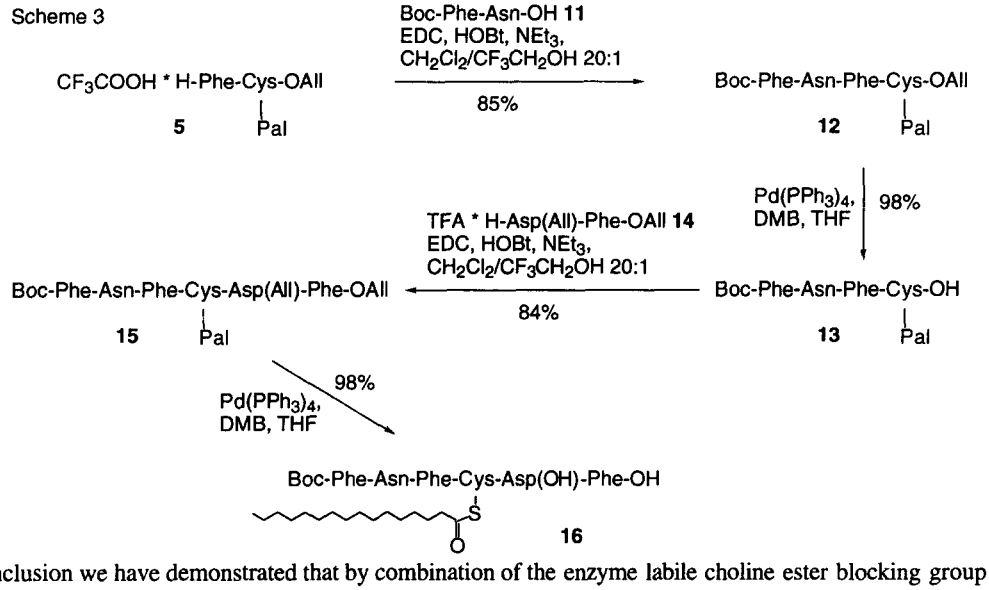


We assume that this cyclic heptasaccharide slips over the palmitoyl residue with its hydrophobic cavity, thereby solubilizing the peptide. Furthermore a beneficial effect may be that the palmitoyl thioester is protected against enzymatic or non- enzymatic hydrolysis after insertion into the cyclodextrin. The cysteinyl peptide **9** is selectively deprotected at the C-terminus and thereby opens up the opportunity to elongate the peptide chain into this direction only and to synthesize various different Y_1-receptor peptides.

Furthermore, the remaining protecting groups could be removed sequentially. Thus, upon treatment with a Pd(0)-catalyst in the presence of dimethylbarbituric acid (DMB),[10] tetrapeptide **10** was formed in high yield. The conditions of this deprotection are so mild that an attack on the thioester does not occur either. Finally, from **14** the Boc group could be cleaved in quantitative yield by treatment with trifluoroacetic acid.

In order to synthesize the palmitoylated hexapeptide **16**, alternatively, a differentiation between the C-terminal carboxylic acid and the aspartic acid side chain is not absolutely necessary, and in particular the allyl ester can be used in this case to mask both acid functions simultaneously.[11] The hexapeptide **16** was synthesized starting with the palmitoylated and N-terminally deprotected dipeptide ester **5** and the Boc-dipeptide **11** as shown in Scheme 3. After coupling of these two building blocks, the allyl ester was removed as described above in a Pd(0)-catalyzed allyl transfer to dimethylbarbituric acid. The tetrapeptide carboxylic acid **13** was then condensed with the aspartyl-phenylalanine bis(allyl ester) **14** which was synthesized from BocAsp(All)OH by coupling with phenylalanine allyl ester and subsequent removal of the Boc group with trifluoroacetic acid. The fully masked hexapeptide **15** obtained thereby was then subjected to a final treatment with $(PPh_3)_4Pd(0)$ and dimethylbarbituric acid to remove both allyl ester blocking functions simultaneously in nearly quantitatively yield.[10] No attack on the palmitic acid thioester was detectable.

Scheme 3



In conclusion we have demonstrated that by combination of the enzyme labile choline ester blocking group with the Pd(0)-sensitive allyl ester very base-labile S-palmitoylated peptides incorporating further carboxy groups can be synthesized selectively. These techniques are appropriate for the synthesis of lipidated peptides representing characteristic partial structures of human Y_1 receptor, which now may serve as new reagents for the study of signal transduction via this G protein coupled membrane bound protein.

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8. **Boc-Phe-Cys(Pal)-Asp(All)-Phe-OH (9):** To a solution of dimethyl-β-cyclodextrin (2.60 g, 1.92 mmol) in phosphate buffer (50 mL, 0.06 M, pH 6.5) was added Boc-Phe-Cys(Pal)-Asp(All)-Phe-OCho (217 mg, 0.20 mmol), and this mixture was sonicated for 20 min. After addition of butyrylcholine esterase (300 units), the mixture was shaken gently at 37° C for 48 h. The pH was adjusted to 2 with 1 M HCl and benzyltriethylammonium chloride (910 mg, 3.84 mmol) was added. The product, which already had precipitated partly during the reaction, was extracted with diethyl ether (5 x 30 mL). After drying the combined organic layers over $MgSO_4$, the solvent was removed under reduced pressure. The product was isolated from the remaining residue by size exclusion chromatography on Sephadex LH 20 with chloroform/methanol (1:1) to yield the product (90 mg, 99 µmol, 49 %) as a white amorphous solid. R_f = 0.4 (hexane/ethyl acetate/acetic acid 50:50:1). $[\alpha]^{22}_D$ = -8.1 (*c* = 0.5, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3/CD_3OD$ 1:1): δ 7.18-7.29 (m, 10H, arom. CH); 5.89 (ddt, *J* = 5.8, 10.5, 17.2 Hz, 1H, C<u>H</u>=CH_2); 5.30 (dd, *J* = 1.5, 17.2, 1H, CH=C<u>H</u>$_{2a}$); 5.21 (dd, *J* = 1.2, 10.4 Hz, 1H, CH=C<u>H</u>$_{2b}$); 4.78 (dd, *J* = 6.2, 6.2 Hz, 1H, α-CH); 4.66-4.69 (m, 1H, α-CH); 4.53-4.59 (m, 2H, OCH_2); 4.42 (dd, *J* = 4.9, 7.6 Hz, 1H, α-CH); 4.24-4.27 (m, 1H, α-CH); 3.21-3.24 (m, 2H, β-CH_{2a} Cys, β-CH_{2a} Phe); 3.08-3.16 (m, 3H, β-CH_{2b} Cys, β-CH_{2b} Phe, β-CH_{2a} Phe'); 2.84-2.89 (m, 2H, β-CH_{2b} Phe', β-CH_{2a} Asp); 2.72 (dd, *J* = 7.1 Hz, 16.8 Hz, 1H, β-CH_{2b} Asp); 2.51-2.57 (m, 2H, α-CH_2 Pal); 1.60-1.63 (m, 2H, β-CH_2 Pal); 1.40 (s, 9H, $C(CH_3)_3$); 1.27 (s, br, 24H, $(CH_2)_{12}$ Pal); 0.89 (t, *J* = 6.9 Hz, 3H, ω-CH_3 Pal).
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10. **Procedure for the removal of allyl esters:**To a solution of the protected peptide in dry THF (20 – 40 mL) was added dimethylbarbituric acid (0.55 equivalents per allyl group) followed by a catalytic amount of tetrakis(triphenyl-phosphine)palladium(0). The reaction was monitored by TLC, and was judged complete with the disappearance of starting material (generally 2 – 3 h). The solvent was then removed under reduced pressure. The product was purified by washing with ether (**15**, **16**) or by flash chromatography (**6**, **10**).
Boc-Phe-Cys(Pal)-Asp-Phe-OH (10): $[\alpha]^{22}_D$ = -27.0 (*c* = 0.9, DMF). ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$ 5:1): δ 7.16-7.38 (m, 10H, arom. CH); 4.71-4.76 (m, 2H, 2 * α-CH); 4.43-4.46 (m, 1H, α-CH); 4.27-4.30 (m, 1H, α-CH); 3.22 (dd, *J* = 5.2, 14.3 Hz, 1H, β-CH_{2a} Cys); 3.07-3.18 (m, 4H, β-CH_{2b} Cys, β-CH_2 Phe, β-CH_{2a} Phe'); 2.86-2.92 (m, 1H, β-CH_{2b} Phe'); 2.78 (dd, *J* = 5.8, 17.2 Hz, 1H, β-CH_{2a} Asp); 2.70 (dd, *J* = 6.2, 17.2 Hz, 1H, β-CH_{2b} Asp); 2.49-2.53 (m, 2H, α-CH_2 Pal); 1.61 (br, 2H, β-CH_2 Pal); 1.39 (s, 9H, $C(CH_3)_3$); 1.25 (s, br, 24H, $(CH_2)_{12}$ Pal); 0.88 (t, *J* = 6.8 Hz, 3H, ω-CH_3 Pal).
Boc-Phe-Asn-Phe-Cys(Pal)-Asp-Phe-OH (16): $[\alpha]^{22}_D$ = -45.3 (*c* = 0.6, DMF). ^{1}H NMR (500 MHz, $CDCl_3/CD_3OD$ 1:1): δ 7.13-7.32 (m, 15H, arom. CH); 4.58-4.70 (m, 3H, α-CH Asn, α-CH Asp, α-CH Phe); 4.42 (br, 2H, α-CH Phe', α-CH Cys); 4.28-4.31 (m, 1H, α-CH Phe''); 3.38-3.42 (m, 1H, β-CH_{2a} Cys); 3.16-3.26 (m, 3H, β-CH_{2b} Cys, β-CH_{2a} Phe, β-CH_{2a} Phe'); 3.09 (dd, *J* = 7.4, 13.9 Hz, 1H, β-CH_{2b} Phe); 2.96-3.04 (m, 2H, β-CH_{2b} Phe', β-CH_{2a} Phe''); 2.88 (dd, *J* = 5.5, 16.9 Hz, 1H, β-CH_{2a} Asx); 2.69-2.82 (m, 4H, β-CH_{2b} Phe'', β-CH_{2b} Asx, β-CH_2 Asx'); 2.56 (t, *J* = 7.5 Hz, 2H, α-CH_2 Pal); 1.36-1.67 (m, 2H, β-CH_2 Pal); 1.36 (s, 9H, $C(CH_3)_3$); 1.26 (s, br, 24H, $(CH_2)_{12}$ Pal); 0.89 (t, *J* = 6.9 Hz, 3H, ω-CH_3 Pal).
11. The use of the choline ester to mask both acid groups turned out to be problematic. Orienting experiments showed that aspartimide formation[9] becomes a major side reaction if the choline ester is used to protect the β-COOH of aspartic acid.