# Chemoselective Acylation of Fully Deprotected Hydrazino Acetyl Peptides. Application to the Synthesis of Lipopeptides

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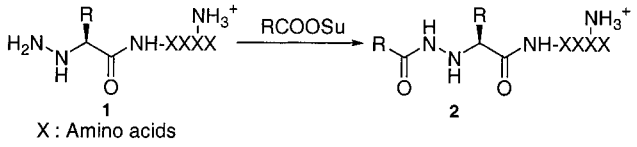
Fully deprotected N-terminal α-hydrazino acetyl peptides were synthesized and chemoselectively acylated on the hydrazine moiety with various fatty acid succinimidyl esters or *N*-(cholesterylcarbonyloxy) succinimide to give lipopeptides of high purity. The buffer and pH were adjusted in order to minimize the oxidation of the hydrazine moiety and to achieve the best conversion and selectivity. The acylation was performed in a citrate−phosphate buffer/2-methylpropan-2-ol mixture of pH 5.1. The p$K_a$ of the α-hydrazino acetyl group on our model peptide was found to be 6.45, i.e., about 2 units lower than the p$K_a$ of a glycyl residue. The reaction was subsequently applied to the synthesis of a 38AA peptide derivatized by a palmitoyl group.

## Introduction

Many papers reporting the derivatization of synthetic peptides by fatty acids, by cholesterol derivatives, and in general by lipophilic moieties have been published during the most recent decade. First of all, this modification is now widely recognized as a means of enhancing the transport across biological membranes[1] or biological barriers such as the blood−brain barrier,[2] the derm,[3] or the intestinal barrier.[4] The modification of a peptide by two lipid chains allows its stable insertion into membranes.[5] The lipidation has also been reported to influence significantly the conformation of peptides[1b,6] or their stability in vivo.[4] Finally, several experiments have revealed that cytotoxic T lymphocyte responses can be induced fairly efficiently by simple lipopeptide vaccines in mice,[7] in primates,[8] and in humans.[9]

**Scheme 1: Acylation of α-Hydrazinopeptides**




However, the development of these applications is hampered by the difficulty in producing lipopeptides, which are sparingly soluble and whose purification is often troublesome. The synthesis of large lipopeptides can be achieved by stepwise solid-phase methods. However, the final cleavage and deprotection step in highly acid media do not allow the modification of peptides by acid-sensitive lipophilic moieties such as unsaturated fatty acids. Thus, the lipophilic moiety cannot easily be modulated, while the nature of the fatty acid is known to have a profound effect upon the interaction with the membrane including its alteration.[10] On the other hand, the lipidation of a separately synthesized and purified peptide or protein is an attractive approach provided that there is a clear discrimination among the different nucleophiles present in the fully deprotected compound.[11] In this context, we have examined the reaction of α-hydrazino acetyl peptides **1** with activated fatty acids in buffered water/2-methylpropan-2-ol mixtures (Scheme 1).[12] We report in this paper that hydrazino peptides **1** were chemoselectively acylated on the hydrazine moiety to give conjugates **2** in good yield and purity. The pH of the solvent mixture and the buffer concentration and composition were optimized to obtain the greatest selectivity. This novel methodology was applied to the syn-

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thesis of a 38 amino acid peptides derivatized by a palmitoyl group.

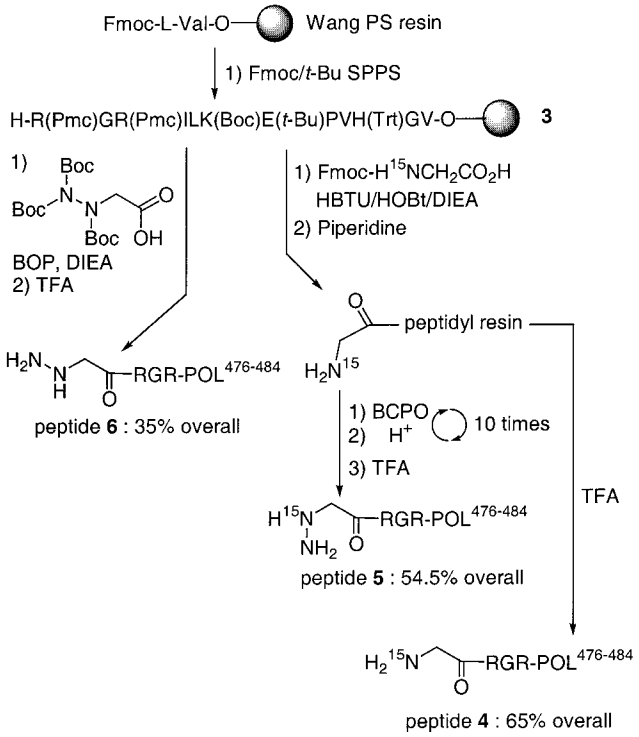
## Results and Discussion

The discrimination between α- and side-chain groups in polypeptides has been the subject of intense research and remains one of the most intractable problems for the peptide or protein chemist. It is rare for the α-amino group of the peptide chain to be distinguishable from its side-chain counterparts, notwithstanding the differences in p*K* between side-chain (p*K*∼10) and terminal groups (p*K*∼8). The acetimidylation reaction, unusually selective for the ϵ-amino group, when compared with the α-amino group, is an exception to this rule.[13] While discrimination is not perfect, the α-amino group tends to be left free to a large and often useful extent, even when ϵ-amino groups are fully substituted.[14] The acylation of peptides is much too difficult to control. For example, reaction of glucagon with acetic anhydride at pH 5.5 gave two products, 70% acetylated only on the α-amino group of the N-terminal His and 30% diacetylated at the N-terminus plus the ϵ-amino group of Lys[12].[15] Desacetylthymosin α1, a 28 amino acid peptide, reacted with acetic anhydride to yield 90% of the N-terminally acetylated peptide,[16] but reaction of trypsinogen with acetic anhydride exhibited little if any selectivity for the N-terminus.[17] For small peptides, with only a single competing lysine group, the reagent iodoacetic anhydride was found to be selective for the N-terminal α-amine at pH 6.[18] However, the selectivity was highly dependent upon both the N-terminal amino acid and the presence/absence of His or Tyr residues. These amino acids are suspected of catalyzing the acylation via the formation of unstable acylimidazoles or phenyl esters. In addition, a large excess of anhydride was used to compensate for the hydrolysis of the reagent.

One way to circumvent these difficulties is to increase the difference in p$K_a$ between the ϵ-amino groups and the nucleophile to be acylated. Working at a pH below 6 should improve the selectivity and minimize histidine or tyrosine residues from acting as acylation catalysts. In addition, lowering the pH should increase the half-life of the activated ester. This latter point is particularly important in the case of activated fatty acids, whose reduced solubility in partial aqueous media preclude the use of large excesses. Some fatty acids are also expensive.

The hydrazino moiety of α-hydrazino acetic acid ethyl ester[19] has a p$K_a$ of 5.97 at 25 °C, i.e., about 2 p*K* units below that of an α-amino group. Interestingly, α-hydrazino acids are known to be regioselectively acylated at the N$\beta$ position, so that only a single isomer is produced.[20] The acylation of α-hydrazino acids should also be less sensitive to the nature of the side-chain, due to the greater distance between the $\beta$-nitrogen and the α-carbon when compared with α-amino acids. Finally, the "α-effect" exhibited by hydrazines was expected to have a favorable impact upon the selectivity.[21] Initially, we have examined the chemoselective acylation of α-hydrazino acetyl peptides **1** (R = H) using the model peptide **6** derived from the POL 476−484 sequence (Scheme 2). Peptide **6** contained a Lys in position 7 for selectivity studies.

**Scheme 2: Synthesis of Peptides 4−6**




**Functionalization of Peptides by an α-Hydrazino Acetyl Group.** The Fmoc/*t*-Bu solid-phase peptide synthesis[22] was used throughout this study since the α-hydrazino acetyl moiety was found to be stable in the concentrated trifluoroacetic acid mixtures employed for the final cleavage and deprotection steps.[23] Owing to the commercial availability of α-hydrazino acetic acid ethyl ester, we focused our attention on the coupling of Boc-protected derivatives of α-hydrazino acetic acid. Monoprotection of α-hydrazino acetic acid on the $\beta$-nitrogen did not suppress the nucleophilic character of the nitrogens and the partial polymerization of the activated ester. Similar observations have been made by Collet et al.[24] On the other hand, (Boc)NHN(Boc)$CH_2CO_2H$ **7** or $(Boc)_2$-NN(Boc)$CH_2CO_2H$ **8** were found to be useful derivatives using either *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)-methylene]-*N*-methylmethanaminium hexafluorophos-

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phate (HBTU)/*N*-hydroxybenzotriazole (HOBt)[25] or benzotriazole-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP)[26] activation. The chemistry related to the preparation, reactivity, and coupling of compounds **7** and **8** will be reported in detail elsewhere.[27] Fully protected derivative **8** was used throughout this study for the preparation of α-hydrazino acetyl peptides. Thus, peptidyl resin **3** (Scheme 2), elaborated starting from Fmoc-L-Val-Wang resin, was acylated with **8** using in situ BOP activation. Model peptide **6** was isolated with a 35% yield following RP-HPLC purification.

**p$K_a$ of the Hydrazino Acetyl Moiety.** The syntheses of peptides **4** and **5** (Scheme 2) were undertaken to permit the determination of the p$K_a$ of the N-terminal amino and hydrazino groups using $^{15}$N NMR spectroscopy. The $^{15}$N-labeled α-hydrazino acetyl moiety of peptide **5** was elaborated on the solid phase by N-electrophilic amination of the N-terminal Gly[$^{15}$N] residue with *N*-Boc-3-(4-cyanophenyl)oxaziridine (BCPO).[28] Reaction of BCPO with a primary amine yields a Boc-protected hydrazine plus an imine resulting from the trapping of the liberated *p*-cyanobenzaldehyde by unreacted amines. The advantage of adopting the solid-phase strategy is based on the selective hydrolysis the imine. Thus, the unmasked amino groups can be rereacted with BCPO following neutralization with diisopropylethylamine. The N-amination/hydrolysis/neutralization cycles are repeated until the disappearance of the amino groups, as confirmed by the use of ninhydrin[29] or 2,4,6-trinitrobenzene-1-sulfonic acid[30] tests. Unhindered primary amines such as the α-amino group of Gly or ϵ-amino groups usually require 5 to 10 cycles. The process was fully automated on a solid-phase peptide synthesizer. Peptides **4** and **5** were isolated with a 65 and 54.5% overall yield, respectively, following RP-HPLC purification.

The variation in the chemical shift of the $^{15}$N nucleus in peptides **4** and **5** as a function of pH is shown in Figure 1. These data corresponded to a p$K_a$ of 6.45 for the α-hydrazino group, and 8.45 for the α-amino group. Thus, as expected, the p$K_a$ of the α-hydrazino acetyl moiety is about 2 p$K_a$ units below that of the α-amino group of Gly.

**Chemoselective Acylation of Peptide 6 with Palmitic Acid Succinimidyl Ester (Scheme 3).** The acylations were performed in a water/2-methylpropan-2-ol: 1/1 mixture, in which both peptides and activated fatty acids proved soluble. Several studies have demonstrated that the addition of an organic solvent to water does not alter

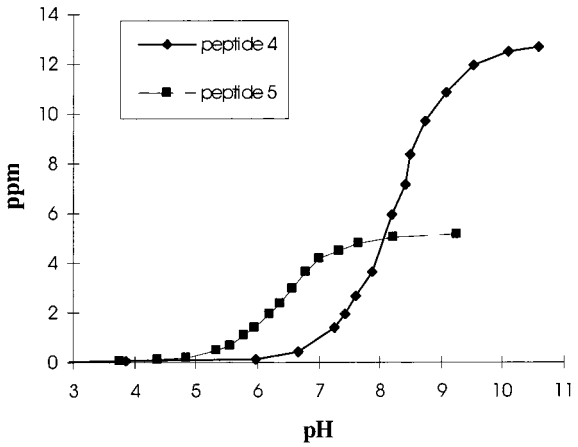
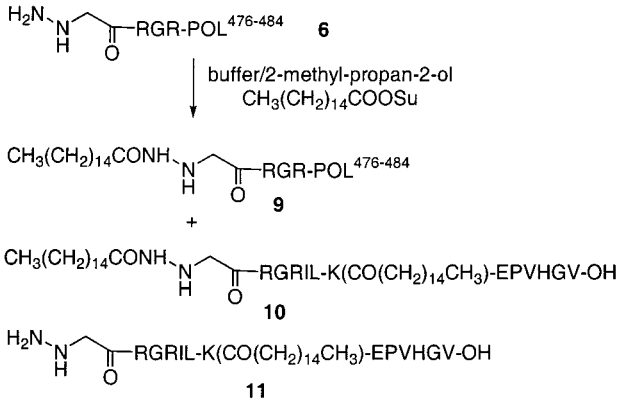



**Figure 1.** Chemical shifts of labeled α-amino (peptide **4**) or hydrazino (peptide **5**) groups as a function of pH at 20 °C ($^{15}NH_3$ was used as an external reference).

**Scheme 3: Reaction of Peptide 6 with Palmitic Acid Succinimidyl Ester**




significantly the p$K_a$ of amines.[31] Thus, the difference in p$K_a$ between the α-hydrazino and amino groups found in water should be conserved upon addition of 2-methylpropan-2-ol. Preliminary experiments were undertaken with a 50 mM citrate−phosphate buffer (pH 5.0, 5.5, and 6.0) using 1.2 equiv of palmitic acid succinimidyl ester on a 1 mg scale. The greatest selectivity and RP-HPLC yield were obtained at pH 5.0. However and unexpectedly, aggregation occurred when the reactions were performed on a larger scale, but this problem was resolved by reducing the concentration of the buffer to 25 mM. The optimal pH was determined more precisely as shown in Figure 2a and 2b using this buffer concentration. Figure 2a corresponds to the RP-HPLC yield for peptide **9**, the lipopeptide resulting from the acylation of the hydrazino moiety. The best yield was obtained at pH 5.11 following 75 h of reaction at room temperature. Figure 2b gives the extent of diacylation, which dramatically increased above pH 5.11. Using these optimal experimental conditions, lipopeptide **9** was isolated with a 69% yield following RP-HPLC purification (6 mg scale, Table, entry 1). Figure 3a shows the RP-HPLC profile of the reaction mixture after 48 h at room temperature. The minor, final eluting peak corresponded to peptide **10** acylated both at the hydrazino and ϵ-amino groups. This side product was isolated with a 6.9% yield following RP-HPLC purification. As expected, the control experiment with the native peptide H-GRGR-POL$^{476-484}$ (peptide **12**) resulted in a low conversion and a poor selectivity (Figure

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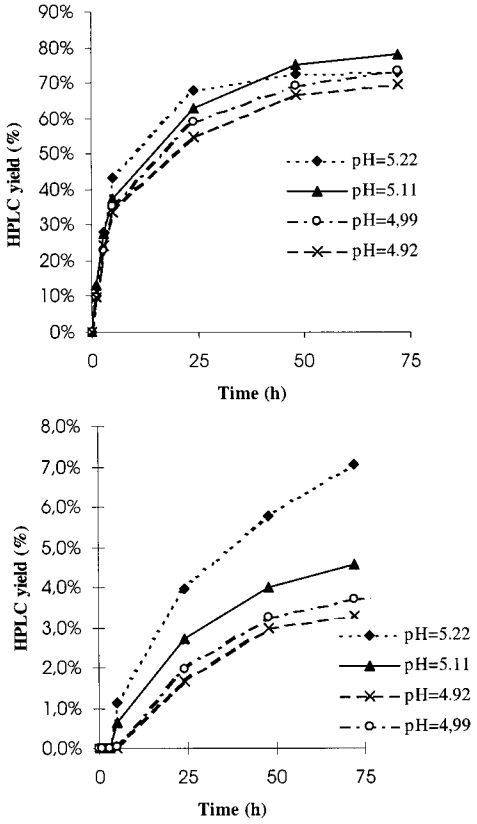


**Figure 2.** (a) RP-HPLC yield for peptide **9** (monoacylation). (b) RP-HPLC yield for peptide **10** (diacylation).

**Table 1. Isolated Yields for Lipopeptides 9 and 14−17**

| entry | peptide | R group | isolated yield (%) |
|---|---|---|---|
| 1 | 9 | palmitoyl | 69 |
| 2 | 14 | oleyl | 49 |
| 3 | 15 | cholesterylcarbonyl | 54 |
| 4 | 16 | *cis*-9,10-epoxystearyl | 31 |
| 5 | 17 | linoleyl | 52 |
| 6 | 18 | stearyl | 47 |

3b). The product resulting from acylation of the N-terminal glycine was isolated with a 28.6% yield, whereas the isomer bearing a palmitoyl group on the $Lys^7$ side-chain represented 8.7%.

Peptide **9** was extensively characterized to ensure the absence of contamination by isomer **11**. Peptide **9** was found to be homogeneous by RP-HPLC and CZE. In addition, the trypsic digest of peptide **9** yielded the expected fragments $CH_3(CH_2)_{14}CONH$-GR-OH, $CH_3$-$(CH_2)_{14}CONH$-GRGR-OH, and ILKEPVHGV-OH, which were isolated and characterized by ES-MS. Fragments corresponding to peptide **11** were not detected.

**Influence of the Buffer.** Recently, Mukerjee et al. have reported that p$K_a$ and pH values of acetate and phosphate buffers are affected differently upon addition of DMSO.[32] 2-Methylpropan-2-ol may have similar effects. Thus, the selectivity of the acylation reaction may depend on the composition of the buffer.

The selectivity of the reaction of peptide **6** with palmitic acid succinimidyl ester was carefully examined in pH 5.1 citrate−phosphate or acetate buffers diluted with 2-methylpropan-2-ol (Figure 4). The RP-HPLC yield for peptide **9** and the selectivity was found to be higher in the

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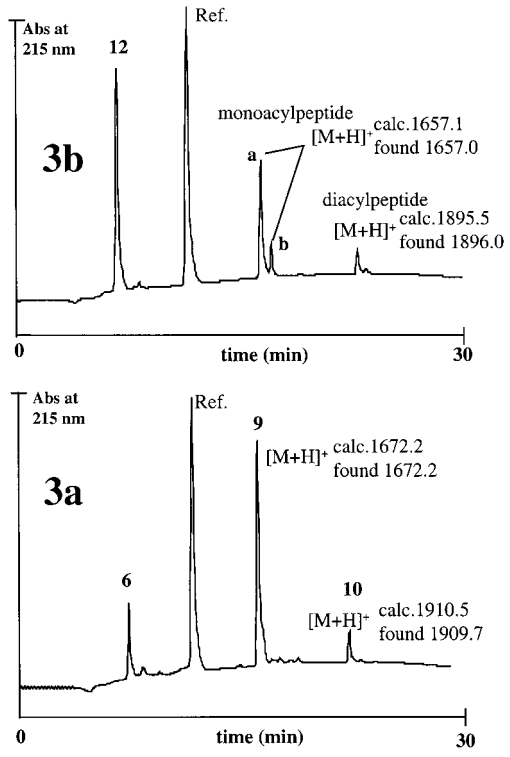



**Figure 3.** (a and b) RP-HPLC of the crude reaction mixtures after 48 h at room temperature, C3 Zorbax column (1 mL/min, 215 nm, linear gradient 0−80% acetonitrile in water in 30 min, 0.05% TFA). (a) peptide **6** $H_2N$-GRGR-POL.$^{476-484}$ (b) control peptide **12** H-GRGR-POL.$^{476-484}$ Peaks a and b corresponded to peptides Palm-GRGRILKEPVHGV-OH and H-GRGRILK-(Palm)EPVHGV-OH, respectively, as determined by ES-MS analyses and Edman degradation.

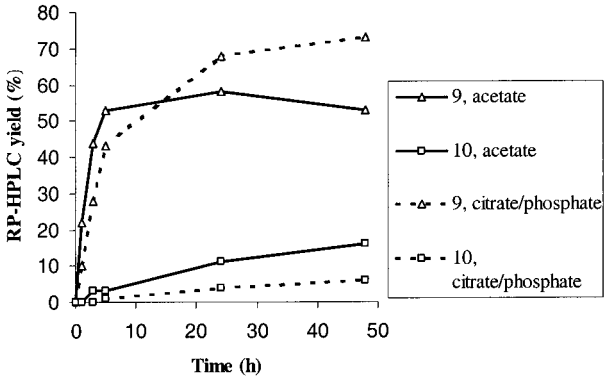



**Figure 4.** Acylation of peptide **6** in citrate/phosphate or acetate buffers.

citrate−phosphate buffer. This result may be correlated to the higher apparent pH of the acetate/2-methylpropan-2-ol mixture (6.36) when compared with the citrate−phosphate/2-methylpropan-2-ol mixture (5.85).

Besides the products resulting from acylation of peptide **6** by palmitic acid succinimidyl ester, the crude reaction mixtures contained minor amounts of a new product, slightly more hydrophobic by RP-HPLC than the starting hydrazino peptide **6**, and which by ES-MS corresponded to Ac-RGR-POL$^{476-484}$ (peptide **13**). This side-product could result from the air oxidation of the α-hydrazino acetyl moiety. The stability of peptide **6** in pH 5.1 citrate−phosphate or acetate buffers was examined (Figure 5). Peptide **6** decomposed exclusively into

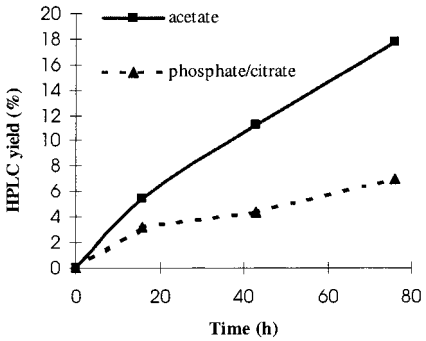
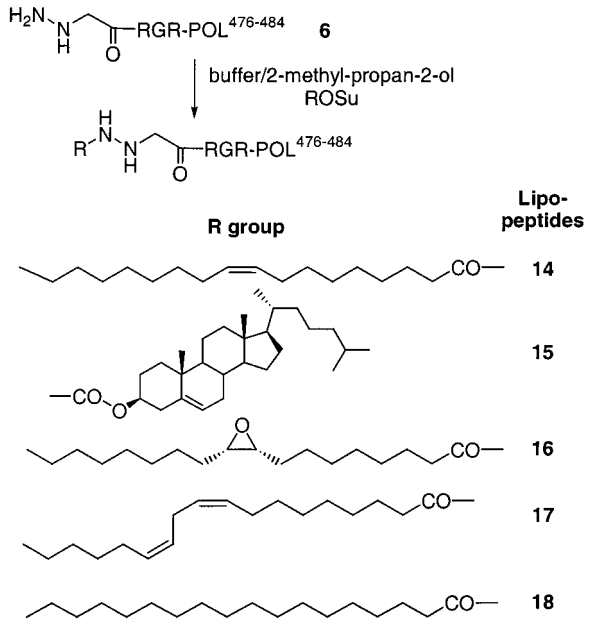



**Figure 5.** Formation of byproduct **13** in 25 mM acetate or citrate−phosphate buffers diluted with 2-methylpropan-2-ol.

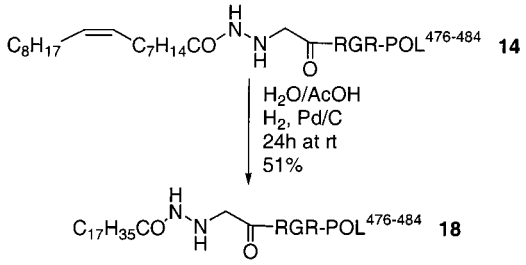
**Scheme 4: Synthesis of Lipopeptides 14−18**



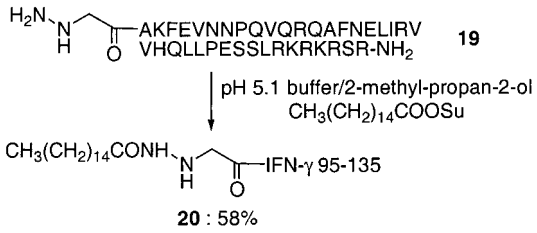

**13** in both media. The RP-HPLC yield for **13** in citrate−phosphate buffer reached 9.6% following 72h at room temperature, but was 3 times as high in acetate buffer. Clearly, the lower yield for lipopeptide **9** in acetate buffer when compared with the citrate−phosphate can be ascribed to both a lower selectivity for the acylation and to a reduced stability of the starting α-hydrazino acetyl peptide.

**Chemoselective Acylation of Peptide 6 with Other Fatty Acid Succinimidyl Esters.** Acylation of peptide **6** with other activated fatty acids or cholesterol derivatives was then examined (Scheme 4, Table). Oleic, linoleic, stearic, and *cis*-9,10-epoxystearic acids were converted into the corresponding succinimidyl esters using diisopropylcarbodiimide/*N*-hydroxysuccinimide in dry THF/$CH_2Cl_2$ at 4 °C. After removal of the solvents in vacuo, the activated esters were redissolved in 2-methylpropan-2-ol and added to the peptide solution. *N*-(Cholesterylcarbonyloxy)succinimide was obtained as a solid following reaction of cholesteryl chloroformate with *N*-hydroxysuccinimide in the presence of triethylamine. These lipophilic derivatives behaved as palmitic acid succinimidyl ester in terms of both reactivity and selectivity. Lipopeptides **14**, **15**, and **17** were isolated with 49−54% yield following RP-HPLC purification. Lipopeptide **18** was found to be identical to the product obtained following catalytic hydrogenation of compound **14** in

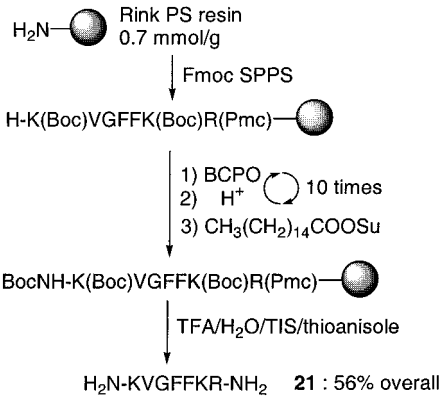
**Scheme 5: Catalytic Hydrogenation of Lipopeptide 14**




**Scheme 6: Acylation of Peptide 19**




**Scheme 7: Synthesis of Peptide 21 by Solid Phase N-Electrophilic Amination**



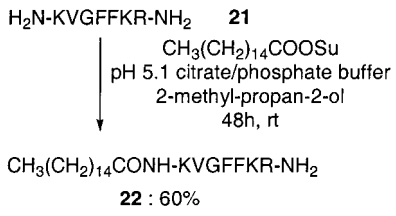

aqueous acetic acid (Scheme 5). The acylation involving *cis*-9,10-epoxystearic acid succinimidyl ester highlights the mildness of the process, since the epoxy moiety is known to be very sensitive to acids. Peptide **16** was purified by RP-HPLC at pH 7.2, since RP-HPLC at pH 2 resulted in the opening of the epoxide (Table, entry 4).

**Application to the Synthesis of Large Lipopeptides.** The application of the methodology to the synthesis of large lipopeptides was examined with peptide **19**, which contained three lysines in its sequence (Scheme 6). Peptide **19** is derived from the murine interferon-$\gamma$ (IFN-$\gamma$) 95−132 sequence. A construct resulting from the modification of the IFN-$\gamma$ 95−132 sequence by a palmitoyl group was found to reproduce the activities of the recombinant IFN-$\gamma$, on both human and mouse cells.[3b]

The acylation of peptide **19** with palmitic acid succinimidyl ester was found to be chemoselective and led to formation of lipopeptide **20** in 58% yield following RP-HPLC purification (Scheme 6). Thus, the methodology could be applied to large α-hydrazino acetyl peptides, despite the presence of several Lys residues in the sequence. However, a scrambled analogue of peptide **19** led to poor results (data not shown), due to aggregation of both the starting material and of the lipopeptide.

**Extension of the Methodology to Other α-Hydrazino Acids.** The solid-phase N-electrophilic amination allows the automated synthesis of peptides func-

**Scheme 8: Acylation of Peptide 21**




tionalized at the N-terminus by an α-hydrazino acid. Thus, the lipidation of such peptides was examined using the model hydrazino peptide **21** (Scheme 7), whose sequence was derived from the 989−995 membrane proximal portion of the GpIIb cytoplasmic tail.[3c] Peptide **21** was synthesized as described in Scheme 7 using standard Fmoc/*t*-Bu chemistry. Following peptide elongation, the α-amino group was reacted with BCPO as described for peptide **5**. Unreacted amino groups were capped with palmitic acid succinimidyl ester. Cleavage in concentrated TFA followed by RP-HPLC purification afforded compound **21** in a 56% yield. Acylation of peptide **21** with palmitic acid succinimidyl ester was found to be very clean, and lipopeptide **22** was isolated with a 60% yield following RP-HPLC purification (Scheme 8).

## Conclusion

The α-hydrazino group of α-hydrazino acids has unique properties when compared with the functional groups present naturally in peptides. Owing to its low p$K_a$, the hydrazino moiety was able to react chemoselectively in aqueous media at pH 5.1 with fatty acid succinimidyl esters, thus giving a very mild access to lipopeptides. The functionalization of peptides by an α-hydrazino acetyl moiety is very easy using a fully protected derivative of α-hydrazino acetic acid. The methodology, which permitted the use of near stoichiometric amounts of the activated ester, was extended to hydrazino lysine. Most of the purification efforts were concentrated upon the isolation of the hydrophilic and soluble hydrazino precursor. Thus, thanks to the very late introduction of the lipophilic tail, lipopeptides of high purity could be isolated with good yields.

## Experimental Section

Solid-phase peptide syntheses were performed using standard Fmoc/*t*-Bu chemistry on Applied Biosystems 430A or 431A or Perseptive Pioneer peptide synthesizers. The amino acids were activated using *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU)/*N*-hydroxybenzotriazole (HOBt)/diisopropylethylamine activation (AA/HBTU/HOBt/DIEA: 4 equiv/ 4 equiv/ 4 equiv/ 8 equiv) in DMF. Side chain protections were as follows: His(Trt), Glu(*t*-Bu), Arg(Pmc), Lys(Boc), Asn(Trt), Tyr(*t*-Bu), Ser(*t*-Bu), Q(Trt). Fmoc amino acids were purchased from Senn Chemicals. Amino acid composition of peptidyl resins was checked by amino acid analysis (Beckman amino acid analyzer model 7300, ninhydrin detection), following hydrolysis of an aliquot in propionic acid/6 N HCl: 1/1 at 140 °C during 3 h.

**Characterization of the Purified Peptides**. The RP-HPLC analyses were performed at 50 °C on C3 Zorbax (4.6 × 250 mm) or C18 Hyperprep (4.6 × 250 mm) columns using water−acetonitrile or water/2-propanol linear gradients. The following buffers were used: Buffer A: water containing 0.05% TFA by vol; Buffer B: acetonitrile/water: 4/1 by vol containing 0.05% TFA by vol; Buffer C: isopropanol/water: 3/2 by vol containing 0.05% TFA by vol; Buffer D: pH 7.2 25 mM triethylamine-phosphate buffer in water; Buffer E: pH 7.2 25 mM triethylamine−phosphate buffer in water/acetonitrile: 1/4 by vol.

Capillary zone electrophoresis (CZE) was performed on an Applied Biosystems 270A-HT apparatus in a 75 μm × 50 cm fused silica capillary, with a 40 mA current and a 30 kV field. The analyses were undertaken in a pH 3.0 sodium citrate buffer unless otherwise stated.

Electrospray mass spectrometry (ES-MS) studies were performed on a Micromass Quatro II Electrospray Mass Spectrometer. The *m*/*z* range 200−2100 was scanned using an ionspray voltage of 4500 V. The orifice plate voltage was ranged between 40 and 60 V. The temperature in the ionization chamber was 60 °C. Time-of-flight plasma desorption mass spectrometry (TOF-PDMS) was undertaken on a Bio-ion 20 Plasma Desorption Mass Spectrometer.

Amino acid compositions were determined using a Beckman amino acid analyzer model 7300 with ninhydrin detection, following hydrolysis in evacuated sealed tubes with 6 N HCl/ phenol: 10/1 (v/v) at 110 °C during 24 h.

**Synthesis of Peptides 4 and 5.** Peptidyl-resin H-R(Pmc)-GR(Pmc)ILK(Boc)E(*t*-Bu)PVH(Trt)GV-Wang-PS was elaborated starting from 0.25 mmol of Fmoc-Val-Wang-PS resin (0.52 mmol/g, Novabiochem). Fmoc-Gly[$^{15}$N]-OH (Euriso-top, 82 mg, 0.275 mmol) was coupled manually using HBTU/DIEA: 104.2 mg (0.275 mmol)/93.9 μL (0.55 mmol) activation in DMF (30 min). The resin was washed with DMF (2 × 2 min), $CH_2Cl_2$ (2 × 2 min), and capped with $Ac_2O$/DIEA/$CH_2Cl_2$: 10/5/85 by vol (2 and 20 min). The resin was then rinsed with $CH_2Cl_2$ (2 × 2 min) and DMF (3 × 2 min) and treated with piperidine/ DMF: 1/4 by vol (2 and 15 min). After rewashing with DMF (1 min), $CH_2Cl_2$ (1 min), and DMF (1 min), half of the resin was taken up in diethyl ether, dried in vacuo, and treated with TFA/water/anisole: 95/2.5/2.5 by vol during 2 h. The crude peptide was precipitated in 100 mL of cold diethyl ether/ heptane: 1/1 by vol, redissolved in water and lyophilized. The crude peptide (224.6 mg) was purified by RP-HPLC on a C18 Hyperprep column (15 × 300 mm) using A and B buffers (0−100% B in 100 min, 3 mL/min, rt, detection at 215 nm). Peptide **4**, 160.7 mg (65%), white powder, ES-MS $[M + H]^+$ calcd 1419.7, found 1418.95. AA(calcd): found; G(3): 2.96, R(2): 2.0, I(1): 0.95, L(1): 1.0, K(1): 1.0, E(1): 1.1, V(2): 2.0, H(1): 0.94.

The other half was subjected to the BCPO procedure (10 cycles) as described elsewere.[30] An aliquot of the peptidyl resin was subjected to the Kaiser test (negative). The resin was washed with $CH_2Cl_2$ and diethyl ether and dried in vacuo. The peptidyl resin was cleaved and deprotected as above to give 228 mg of a white powder. The crude peptide was purified by RP-HPLC on a C18 Hyperprep column (15 × 300) using buffers A and C (0−60% C in 60 min, 3 mL/min, rt, detection at 215 nm). Peptide **5**, 136 mg (54.3%), white powder, ES-MS $[M + H]^+$ calcd 1434.7, found 1434.0. AA(calcd): found; G(2): 2.0, R(2): 2.0, I(1): 0.96, L(1): 1.0, K(1): 1.0, E(1): 1.1, V(2): 2.0, H(1): 0.87.

***p*$K_a$ Determination.** The p$K_a$ of the α-amino group of peptide **4** and of the α-hydrazino group of peptide **5** were determined using $^{15}$N NMR spectroscopy on a Bruker DRX300 NMR spectrometer at 20 °C. Peptides **4** and **5** were dissolved in $D_2O$ at a concentration of 97.7 and 96.9 mg/mL, respectively. The pH was adjusted with 1 N aqueous NaOH. Chemical shifts were referenced relative to external $^{15}NH_3$.

**Synthesis of $H_2$N-GRGR-POL$^{476-484}$-OH Peptide (peptide 6).** Peptide **6** was elaborated starting from 0.125 mmol of Fmoc-L-Val-Wang PS resin (0.73 mmol/g, Applied Biosystems, Foster City, CA). Following peptide elongation, $(Boc)_2N-N(Boc)CH_2CO_2H$ (58.5 mg, 0.15 mmol) was coupled twice manually using BOP (66.3 mg, 0.15 mmol)/DIEA (78.4 μL, 0.45 mmol) activation in DMF (30 min). The peptidyl-resin was washed with DMF, $CH_2Cl_2$, and diethyl ether and dried in vacuo. Cleavage and deprotection were performed using 10 mL of a TFA/water/anisole: 95/2.5/2.5 by vol mixture during 2 h at room temperature. The crude peptide was precipitated in cold diethyl ether/heptane: 1/1 by vol, redissolved in water/ acetic acid: 5/1 by vol, and lyophilized. Purification was performed on a C18 Hyperprep (15 × 300 mm) column using

buffers A and C (0−50% C in 100 min, 3 mL/min, rt, detection at 215 nm). The pure fractions were pooled and lyophilized to give 84.2 mg (34%) of a white powder. AA(calcd): found; G(2): 1.9, R(2): 2.0, I(1): 1.0, L(1): 1.2, K(1): 1.0, E(1): 1.6, V(2): 2.0, H(1): 0.9. ES-MS: M calcd 1432.7, found 1433.0.

**Activation of Fatty Acids, Typical Experimental Procedure**. Oleic acid (4.96 mg, 17.5 $\mu$moles) was dissolved in 175 $\mu$L of THF/$CH_2Cl_2$: 1/1 by vol 17.7 $\mu$L (17.5 $\mu$mol) of *N*-hydroxysuccinimide 0.99 M in THF and 15.0 $\mu$L (13.5 $\mu$mol) of diisopropylcarbodiimide 0.90 M in $CH_2Cl_2$ were added at 4 °C. The reaction mixture was stirred overnight. The solvents were then removed in vacuo, and the residual oil was redissolved in 600 $\mu$L of 2-methylpropan-2-ol and immediately used in the acylation reactions.

**Preparation of *N*-(Cholesterylcarbonyloxy)succinimide.** Cholesteryl chloroformiate (Janssen, 500 mg, 1.13 mmol) and *N*-hydroxysuccinimide (140.9 mg, 1.22 mmol) were dissolved in 2 mL of $CH_2Cl_2$ at room temperature. Triethylamine (170 $\mu$L, 1.22 mmol) was added in one portion. The reaction was stirred 45 min, diluted with 50 mL of $CH_2Cl_2$, and washed with water saturated with $KH_2PO_4$. The organic phase was dried over sodium sulfate. Removal of the solvents under reduced pressure yielded 451.6 mg (76%) of a white powder which was used without further purification. $^1$H NMR (300 MHz, DMSO-$d_6$, 296 K) $\delta$ 5.41 (1H, m), 4.60 (1H, m), 2.82 (4H, s), 2.48 (2H, m), 2.04−0.85 (38H, m), 0.68 (3H, s). $^{13}$C NMR (150 MHz, DMSO-$d_6$, 296 K) $\delta$: 169.8, 151.7, 139.4, 124.8, 82.7, 57.0, 56.5, 50.2, 42.6, 39.9, 39.8, 37.8, 36.9, 36.4, 36.0, 32.1, 28.4, 28.2, 27.6, 25.6, 24.4, 24.0, 22.9, 22.7, 21.2, 19.3, 28.8, 11.9.

**Acylation of Peptide 6. Typical Experimental Procedure.** Peptide **6** (6.0 mg, 3.0 $\mu$mol) was dissolved in 900 $\mu$L of a citrate−phosphate pH 5.2 buffer. The pH of the solution was adjusted to 5.1 with a 0.2 M $Na_2HPO_4$ aqueous solution. Palmitic acid succinimidyl ester (Sigma, 1.16 mg, 3.3 $\mu$mol) was dissolved in 900 $\mu$L of 2-methylpropan-2-ol and added to the peptide solution. The reaction was monitored by RP-HPLC on a C3 Zorbax column using buffers A and B (0−100% B in 30 min then 5 min at 100% B, 1 mL/min, 50 °C, detection at 215 nm). Following 72 h at room temperature, the reaction mixture was diluted with 5 mL of water/acetic acid: 4/1 by vol and purified on a C3 Zorbax column (0−60% B in 70 min, 3 mL/min, 50 °C, detection at 215 nm). Peptide **9**, 4.41 mg (69%), white powder, MALDI-TOF $[M + H]^+$ calcd 1672.2, found 1672.2.

Peptides **14**/**15** and **17**/**18** were synthesized and purified similarly.

Starting from 5.02 mg of peptide **6**, 2.58 mg (49%) of peptide **14** was obtained, white powder, ES-MS $[M + H]^+$ calcd 1697.2, found 1697.0.

Starting from 5.04 mg of peptide **6**, 2.27 mg (54%) of peptide **15** were obtained, white powder, ES-MS $[M + H]^+$ calcd 1845.6, found 1845.6.

Starting from 5.07 mg of peptide **6**, 2.83 mg (52%) of peptide **17** were obtained, white powder, ES-MS $[M + H]^+$ calcd 1695.1, found 1695.5.

Starting from 5.01 mg of peptide **6**, 2.53 mg (47%) of peptide **18** were obtained, white powder, ES-MS $[M + H]^+$ calcd 1699.2, found 1699.7.

Peptide **16** was purified on a C3 Zorbax column using buffers D and E (0−60% E in 70 min, 3 mL/min, 50 °C, detection at 215 nm). Starting from 4.96 mg of peptide **6**, 1.67 mg (31%) of peptide **16** were obtained, white powder, ES-MS $[M + H]^+$ calcd 1713.1, found 1713.1.

**Synthesis of Peptide 19.** Peptide elongation was performed starting from 0.2 mmol of a Fmoc-PAL-PS resin (0.16 mmol/g, Perseptive). Then, an aliquot of the peptidyl resin (127.3 $\mu$mol) was acylated with $(Boc)_2N-N(Boc)CH_2CO_2H$ as described previously for peptide **6**. Cleavage and deprotection were achieved using TFA/water/phenol/ethanedithiol/thioanisole: 10 mL/0.5 mL/0.75 g/0.25 mL/0.5 mL during 2 h at room temperature. The crude peptide was precipitated in diethyl ether/heptane: 1/1 by vol, dissolved in water/acetic acid: 5/1 by vol and lyophilized. RP-HPLC purification was performed on a C3 Zorbax (15 × 300 mm) column using buffers A and C (25−70% C in 60 min, 3 mL/min, 50 °C, detection at 215 nm). Following lyophilization, 126 mg (17%) of peptide **19** were obtained as a white powder. AA(calcd): found; R(6): 6.0, K(3): 2.8, E(7): 7.3, A(2): 1.9, L(4): 4.0, S(3): 2.7, F(2): 1.8. ES-MS: $[M + H]^+$ calcd 4645.4, found 4644.5.

*Synthesis of Lipopeptide **20** by Acylation of **19** with Palmitic Acid Succinimidyl Ester*. 8.15 mg (1.4 $\mu$mol) of peptide **19** were reacted with palmitic acid succinimidyl ester as described previously. The crude peptide was purified by RP-HPLC on a C3 Zorbax (15 × 300 mm) column using buffers A and B (0−60% C in 80 min, 3 mL/min, 50 °C, detection at 215 nm) to give 4.79 mg (57.8%) of a white powder. ES-MS: $[M + H]^+$ calcd 4883.8, found 4882.5.

**Synthesis of Peptide 21.** Peptide elongation was performed starting from 0.25 mmol of Rink amide AM-PS resin (0.70 mmol/g, Senn Chemicals). Then, the peptidyl resin was subjected to the BCPO procedure (10 cycles), neutralized with diisospropylethylamine/$CH_2Cl_2$: 1/19 by vol (2 × 2 min), and capped with palmitic acid succinimidyl ester in DMF. The resin was washed with diethyl ether (2 × 2 min) and dried in vacuo. The final cleavage and deprotection steps were performed using 10 mL of TFA/water/thioanisole/TIS: 94/2.5/2.5/1 by vol during 1 h 30 min. The crude peptide was precipitated in cold diethyl ether/pentane: 1/1 by vol, dissolved in water/acetic acid: 9/1 by vol, and lyophilized. The RP-HPLC purification was performed on a C3 Zorbax (15 × 300 mm) column using buffers A and C (0−70% C in 70 min, 2 mL/min, 30 °C, detection at 215 nm) to give 124 mg (56%) of a white powder. TOF-PDMS: $[M + H]^+$ calcd 896.1, found 895.9. AA(calcd): found; G(1): 0.94, V(1): 1.0, F(2): 2.0, K(1): 1.0, R(1): 1.0.

**Synthesis of Peptide 22.** Peptide **21** (5.06 mg, 3.75 $\mu$mol) was reacted with palmitic acid succinimidyl ester as described previously. Following 48 h at room temperature, the reaction mixture was purified by RP-HPLC on a C3 Zorbax column using A and B buffers (0−100% B in 90 min, 3 mL/min, 30 °C, detection at 215 nm) to give 3.26 mg (60%) of a white powder. ES-MS: $[M + H]^+$ calcd 1133.9, found 1133.8. AA(calcd): found; G(1): 0.99, V(1): 1.03, F(2): 2.0, K(1): 1.02, R(1): 0.97.

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**Supporting Information Available:** $^1$H and $^{13}$C NMR of *N*-(cholesterylcarbonyloxy)succinimide. RP-HPLC, mass spectra, amino acid analysis, and capillary zone electrophoresis of peptides **4**−**6**, **9**, **14**. RP-HPLC, mass spectra, and capillary zone electrophoresis of peptides **16**, **17**, **19**, and **20**. RP-HPLC, mass spectra and amino acid analysis of peptides **21** and **22**. RP-HPLC and mass spectra of peptides **15** and **18**. This material is available free of charge via the Internet at http://pubs.acs.org.

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