Site-Specific Cleavage—A Model System for the Identification of Lipid-Modified Glutamate Residues in Proteins

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Numerous proteins are modified post-translationally after their biosynthesis at the ribosomes of the cell. One such modification, only poorly characterized to date, is the formation of lipid esters of glutamate side chains in the skin proteins of land-living mammals; here a subset of very long chain fatty acids, ceramides and/or glucosylceramides, are bound through their ω-hydroxy groups to structural proteins of the so-called "cornified envelope" in the outermost layer of the skin, the stratum corneum. *We report an approach for the identification of proteins containing ester-modified glutamic acid residues and the determination of their positions within the peptide sequence, designed for mass spectrometric investigation of human skin proteins.*

Introduction

After their biosynthesis at the ribosomes of the cell, numerous proteins are modified post-translationly. One post-translational modification, which is only poorly characterized to date, is the formation of lipid esters of glutamate side chains in the skin proteins of land-living mammals. A subset of very long chain fatty acids, ceramides[1,2] and glucosylceramides,[3,4] are bound through their ω-hydroxy groups to structural proteins of the so-called "cornified envelope" in the outermost layer of the skin, the *stratum corneum*. Because of their high degrees of cross-linking by isodipeptide and disulfide bonds and the great heterogeneity within the lipid parts, neither the identities of the modified proteins nor the positions of the altered glutamate residues are well established.

Here we report an approach to the identification of proteins containing ester-modified glutamic acid residues and the determination of their positions within the peptide sequence. With the aid of model peptides, we have developed conditions for the selective cleavage of the peptide-bond N-terminal of glutamic acid esters, through an approach related to the affinity-cleavage technique.[5] The method was designed to determine such positions in human skin proteins by mass spectrometry.

In human-skin proteins, post-translationally modified glutamic acid side chains are found linked through ester moieties to ω-hydroxylated lipids. This modification is essential for the function of the skins of land-living mammals. Scheme 1 shows a representative structure of a protein-bound glucosylceramide (A), a transient intermediate in the formation of protein-bound lipids, which has only been identified in skin samples of genetically engineered mice with defective glucosylceramide degradation.[3,4] In addition, Scheme 1 also shows the structures of a protein-bound skin ceramide (B) and an ω-hydroxylated fatty acid (C).

Structural alterations of the γ-carboxy groups of glutamic acid side chains in proteins are rare post-translational events: proteins of prokaryotic organisms can contain methyl esters;[6] however, while eukaryotic proteins can be methylated at different positions, this modification of a glutamate side chain has not been found in higher organisms.[7]

Thioesters of glutamate side chains with internal cysteine residues occur in α2-macroglobulin,[8–10] in complements C3 and C4[11–13] and in succinyl-CoA:3-keto acid transferase.[14]

A remarkable feature of all these glutamic acid ester- or thioester-containing proteins is that they become labile with respect to fragmentation on elevation of the pH. In addition, a 2-deoxyribosyltransferase chemically modified by affinity labelling became labile with respect to alkaline hydrolysis after esterification of the catalytic glutamic acid side chain.[15]

The instability of proteins containing glutamic acid esters or thioesters towards hydrolysis has been attributed to the formation of internal pyroglutamate residues (Schemes 2 and 3). Such 1-acylpyrrolidine-2-ones are diacylamines, and it has been known for a long time that substances of this type are readily hydrolysed by dilute aqueous alkaline solution.[16] The chemical conversion of glutamic acid residues into 1-acylpyrrolidine-2-ones with thionyl chloride and pyridine has been proposed as a method for the specific cleavage of peptides at the N-terminal site of glutamate residues.[17–20] In this contribution, we report on the development of conditions that make use of this instability as an analytical tool for the mass spectrometric identification of ester-modified glutamic acid residues in peptides.

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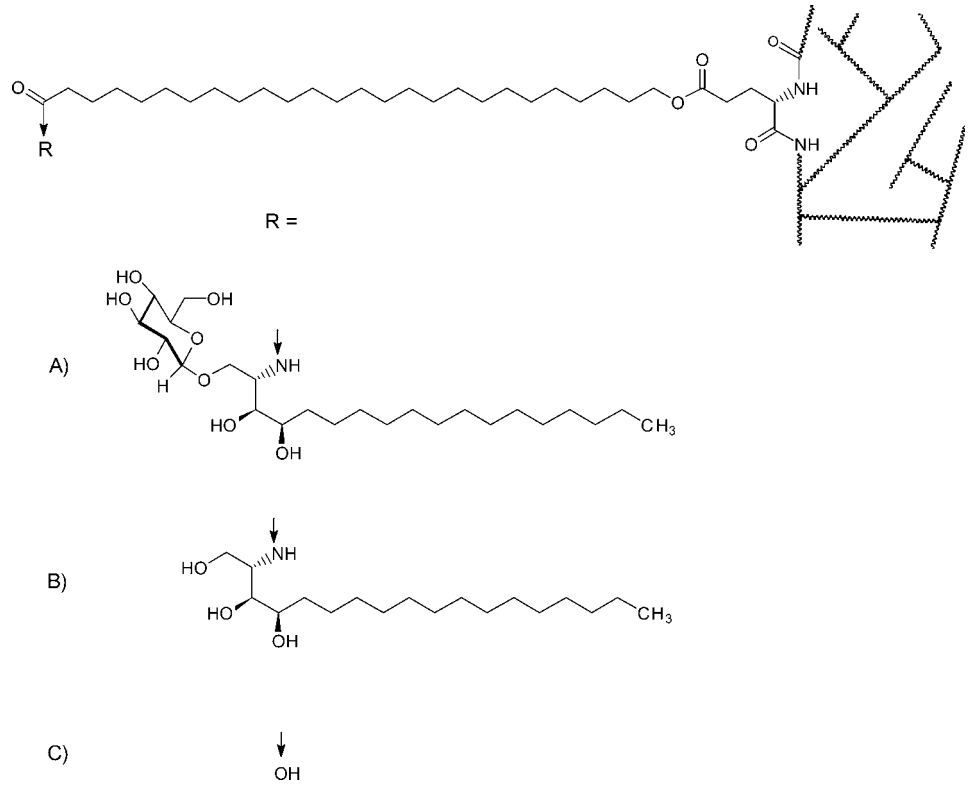
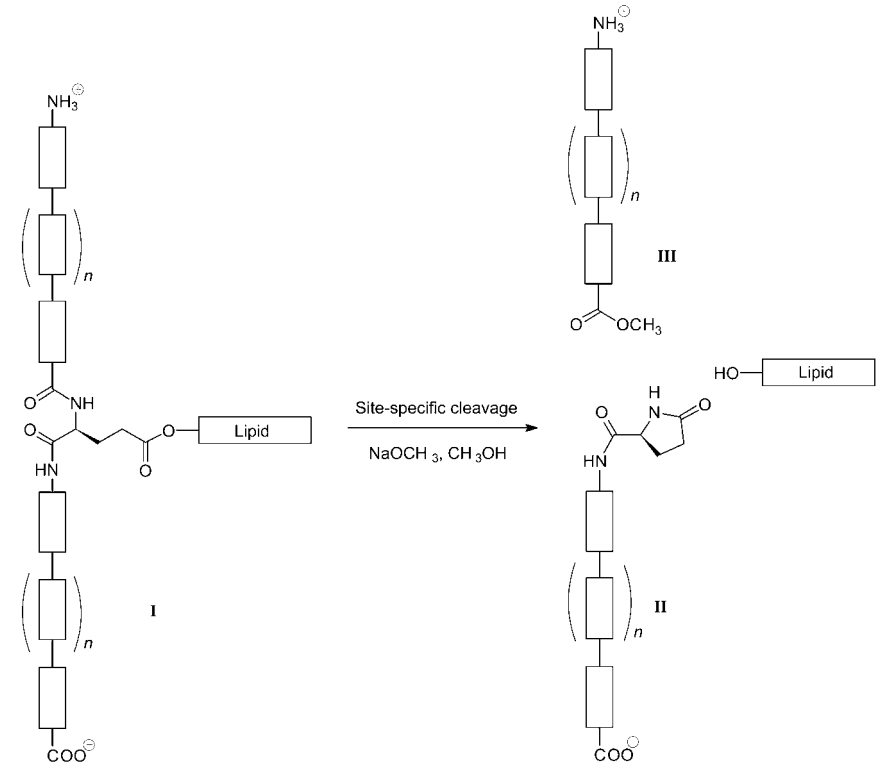


Scheme 1. *Schematic structures of ω-hydroxylated A) glucosylceramide, B) ceramide and C) fatty acid covalently bound to glutamic acid side chains in proteins of the cornified envelope of human skin.*



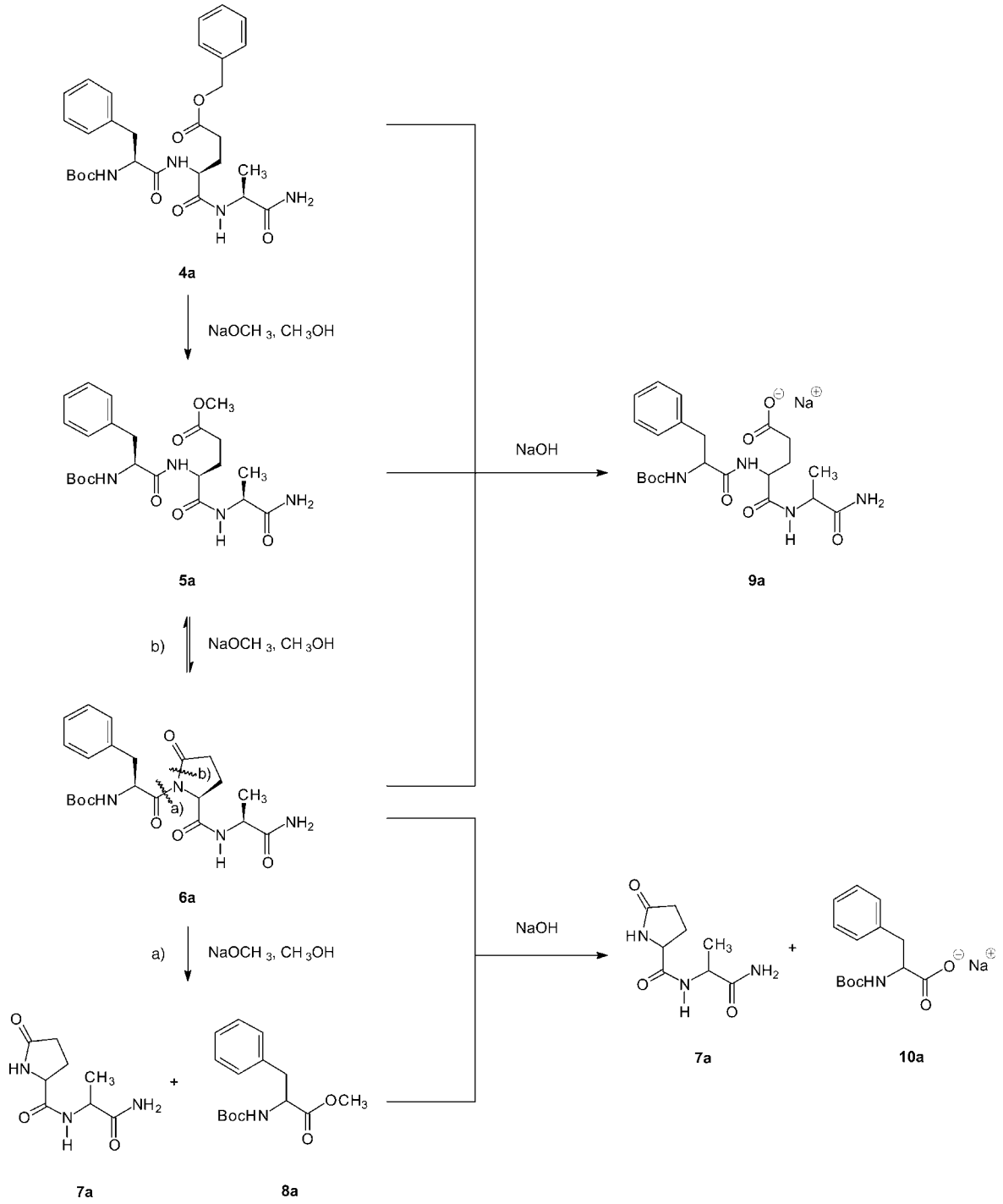
Scheme 2. *Schematic representation of the site-specific cleavage for the identification of esterified glutamate residues in peptides. Vertical boxes represent amino acid residues. Under the given conditions, glutamic acid ester-containing peptides such as* ***I*** *are cleaved into diagnostic products: the peptides* ***II*** *and* ***III*** *and the formerly ester-linked lipid.*

This should also allow the determination of the positions of ω-hydroxylated lipids in human skin proteins.

Our concept for the identification of glutamic acid ester-containing peptides is outlined in Scheme 2. Adaptation of the approaches of Battersby et al.[17–19] and of Clayton et al.[20] to the selective cleavage of peptides **I** containing glutamic acid esters requires conditions under which this structural element is cleaved. The diagnostic cleavage products are peptides **II**, with N-terminal pyroglutamic acid residues, and peptides **III**, with C-terminal methyl esters (see Scheme 3). These peptides can be distinguished from unmodified peptides by electrospray mass spectrometry. In addition, the ω-hydroxylated lipids are also liberated during the procedure.

Since the information about the ester-modified glutamic acid residues is lost during saponification (see below), we developed a protocol that, on the one hand, avoids the use of aqueous media and, on the other, ensures selective peptide cleavage.

A more detailed description of the concept is given in Scheme 3. For the case of one of our model peptides (**4a**) as an example, it shows the selective cleavage of the peptide bond N-terminal of the central glutamic acid ester residue (cf Table 1 for peptide structures). Treatment of **4a** with sodium methoxide in methanol should lead to a transesterification reaction, giving rise to the methyl ester **5a**, and to formation of the peptide derivative **6a** with an internal acylpyrrolidinone moiety. Compound **6a** can be cleaved at two positions, indicated by a) and b) in Scheme 3. Methanolysis of **6a** in position a) should lead to formation of peptide **7a** with an N-terminal pyroglutamyl residue. Compound **7a** represents the



Scheme 3. *Reaction of glutamic acid ester* ***4a*** *in the presence of sodium methoxide in methanol and traces of water. The reactions leading to affinity cleavage are shown in the vertical direction. Reaction of traces of water with* ***4a***, ***5a*** *and* ***6a*** *according to cleavage modus b) leads to a loss of information. With time, the cleavage product* ***8a*** *is converted into* ***10a*** *in the presence of water.*

diagnostic peptide **II** in Scheme 2, which corresponds to the C-terminal part of the initially applied peptide. In addition, the N-terminal part of the former peptide **I** (**III** in Scheme 2) is liberated as the methyl ester **8a**.

Cleavage of **6a** can also proceed according to mode b). Although nucleophilic attack on the more electrophilic carboxy group according to a) should be favoured over ring-opening corresponding to cleavage mode b),[17–20] the latter reaction is unwanted if hydroxide is the nucleophile and should be repressed by the reaction conditions. If methoxide is the nucleophile, b)-mode cleavage leads to regeneration of **5a**, while if hydroxide is the nucleophile it results in the formation of glutamic acid-containing peptides **9a** with loss of information about the modification. Since the presence of water cannot be completely avoided when peptide preparations derived from living tissues are used, it has to be assumed that hydrolysis in-

Table 1. *Structures of model peptides. The HPLC solvent system used (solvent systems I–III) is as specified in the Experimental Section (General).*

Number	Sequence	HPLC solvent system
1	Boc-Glu(OBn)-OH	
2	Boc-Glu(OBn)-Ala-NH_2	
3	H-Glu(OBn)-Ala-NH_2	
4a	Boc-Phe-Glu(OBn)-Ala-NH_2	I
4b	Boc-Val-Glu(OBn)-Ala-NH_2	I
4c	H-Thr-Tyr-Ile-Cys(SBn)-Glu(OBn)-Val-Glu-Asp-Gln-Lys-Glu-Glu-OH	II
4d	Boc-Phe-Glu($OC_{12}H_{25}$)-Ala-NH_2	I
4e	H-Gln-Phe-Phe-Gly-Leu-Met-NH_2	III
5a	Boc-Phe-Glu(OMe)-Ala-NH_2	I
5b	Boc-Val-Glu(OMe)-Ala-NH_2	I
5c	H-Thr-Tyr-Ile-Cys(SBn)-Glu(OMe)-Val-Glu-Asp-Gln-Lys-Glu-Glu-OH	II
7a/b	Pyroglu-Ala-NH_2	I
7c	Pyroglu-Val-Glu-Asp-Gln-Lys-Glu-Glu-OH	II
8a	Boc-Phe-OMe	I
8b	Boc-Val-OMe	I
8c	H-Thr-Tyr-Ile-Cys(SBn)-OMe	II
9a	Boc-Phe-Glu(OH)-Ala-NH_2	I
9b	Boc-Val-Glu(OH)-Ala-NH_2	III
10a	Boc-Phe-OH	I
10b	Boc-Val-OH	I
10c	H-Thr-Tyr-Ile-Cys(SBn)-OH	I

stead of methanolysis of **6a** according to cleavage mode b) (Scheme 3) leads to the formation of fragment **9a**. In addition, it has to be expected that one of the fragments, the methyl ester **8a**, can be converted into the acid **10a** in the presence of water.

The reaction of the methyl ester **5a** with hydroxide ions thus gives rise to the undesired formation of **9a**. Therefore, we decided to conduct the cleavage reaction under conditions that avoid the presence of water as much as possible.

Results and Discussion

To investigate whether the concept outlined in Schemes 2 and 3 would be suitable for the determination of ester-modified glutamic acid ester residues by site-specific cleavage, we decided to start the investigation using tripeptide derivatives containing a central glutamic acid bearing a benzyl ester group in the side chain. The benzyl alcohol represents a model for the lipid residue of the modified skin protein. The use of more complex model peptides turned out to be inappropriate for HPLC investigations, since we observed nearly complete racemization of the peptide samples under the cleavage conditions (Figure 1). This is no disadvantage of the method, since peptide fragments derived from skin samples are detected by mass spectrometry, but it prevents precise quantification of cleavage products by HPLC.

We prepared the model peptides Boc-Phe-Glu(OBn)-Ala-NH_2 (**4a**) and Boc-Val-Glu(OBn)-Ala-NH_2 (**4b**; Table 1) by solution methodology. Boc-Glu(OBn)-OH (**1**) was activated either with dicyclohexylcarbodiimide or with *N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) with the use of *N*-hydroxybenzotriazole (HOBt) as additive[21–23] and coupled to H-Ala-NH_2. Deprotection of Boc-Glu(OBn)-Ala-NH_2 (**2**) with trifluoroacetic acid to provide H-Glu(OBn)-Ala-NH_2 (**3**) was followed by carbodiimide/HOBt-mediated coupling to Boc-Phe-OH or Boc-Val-OH. We applied the model peptides to the cleavage conditions in the same way as we did peptides derived from *stratum corneum* preparations from the skin of human individuals.[2] At concentrations of 1.5–2.0 mg mL^{-1}, the model peptides **4a** and **4b** were treated with sodium methoxide solution in methanol (0.25 M) at room temperature for different time periods. The reaction products were separated by HPLC (Figure 1) and identified by electrospray mass spectrometry (ESI-MS). The kinetics of product formation were investigated by HPLC analysis at different time points and quantified by correlation of the UV absorption with those of known concentrations of the chemically prepared intermediates. For this purpose, and to obtain supporting analytical data (^{1}H NMR, ^{13}C NMR spectra), the cleavage products were synthesized on a preparative scale.

HPLC analysis of the time courses of the transesterifications of **4a** (Figure 2) and **4b** showed that the benzyl esters **4a** and **4b** (Scheme 3) were no longer detectable after only one minute under the conditions described in the Experimental Section. This was also indicated by TLC separation of the reaction mixtures (staining with ninhydrin or molybdophosphoric acid). HPLC investigation of the products formed on treatment of **4a** and **4b** with sodium methoxide in methanol revealed that the methyl esters **5a** and **5b** had been formed in nearly quantitative amounts after 30 minutes reaction time (Figure 2). Prolonged treatment (24 h) with sodium methoxide in methanol led to nearly quantitative fragmentation of the peptide methyl esters **5a** and **5b**. The half-lives of the methyl esters varied (we found half-lives in a range from 2 to 7 h), which we attribute to different amounts of water present in the different preparations. Configurational integrity in the cleavage prod-

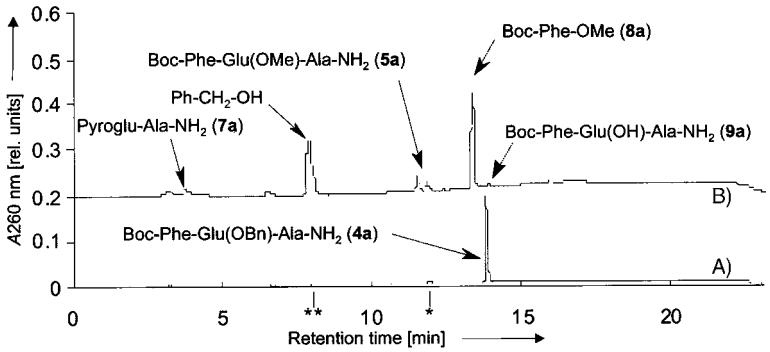


Figure 1. *HPLC chromatogram of peptide **4a**: A) without treatment with sodium methoxide, and B) after 16 h treatment with sodium methoxide in methanol and subsequent neutralization as described in the Experimental Section. Structures and numbers of the identified products are given. Peptide **4a** was dissolved in methanol, and trace amounts of methyl ester **5a** (*) and liberated benzyl alcohol (**) are therefore already detectable in sample A. In B, multiple peaks were observed for **5a** due to racemization.*

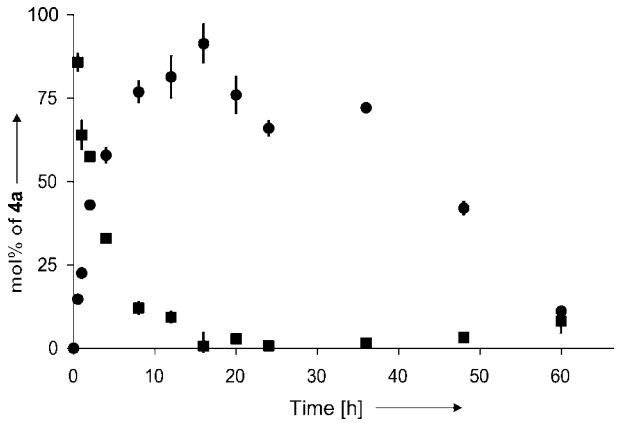


Figure 2. *Time courses of the formation and conversion of methyl ester* ***5a*** *(squares) and fragment* ***8a*** *(circles) on treatment of benzyl ester* ***4a*** *with a 0.25% solution of sodium methoxide in methanol at room temperature. Formation of* ***5a*** *from* ***4a*** *is complete within a few minutes,* ***5a*** *having been formed in quantitative yield after 30 minutes (first data point). The symbols represent the mean value of two independent experimental series, and the bars indicate the range of the determined values.*

ucts **7a**, **7b**, **8a** and **8b** was lost, as determined by ^{1}H NMR spectroscopy, optical rotation or the occurrence of multiple products with identical mass as determined by ESI-MS, but with different HPLC retention times.

After 60 h reaction time under these conditions, complete conversion of the cleavage product **8a** was observed (Figure 2). This is consistent with the assumption of a slow saponification of the methyl ester by hydroxide ions generated by small amounts of water during the incubation, giving rise to the product **10a** (Scheme 3).

Investigation of potential acidic cleavage conditions revealed that no cleavage occurred with 70% formic acid (*v*/*v*) or with trifluoroacetic acid at room temperature over 24 h. Formation of β-peptides, which would be generated through cleavage of a 2-oxopiperidine intermediate formed by attack of the amide nitrogen of the amino acid next to the C terminus of glutamate,[17] also could not be detected. Related reaction behaviour has been observed on treatment of aspartyl-containing peptides under acidic and alkaline conditions.[24]

As an additional model peptide of more complex structure, H-Thr-Tyr-Ile-Cys(SBn)-Glu(OBn)-Val-Glu-Asp-Gln-Lys-Glu-Glu-OH (**4c**; Table 1), was investigated for transesterification and cleavage under the reported conditions. Complete conversion of the starting material was observed after 30 minutes, and after 24 h the formation both of the N-terminal pyroglutamyl-containing cleavage product (**7c**) and of the corresponding C-terminal fragment (**8c**) was demonstrated by mass spectrometry. Precise quantification of the reaction products, the transesterification product **5c** and of the cleavage products **7c** and **8c**, by HPLC was not possible, due to the formation of stereoisomers with overlapping HPLC retention times. In addition, an elimination product containing a dehydroalanine moiety instead of the benzyl-protected cysteine residue was formed. However, estimation from HPLC chromatograms indicates that the reaction kinetics of **4c** are comparable to those of the model peptides **4a** and **4b**.

To ensure that the identity of the alcohol moiety linked to the glutamate residue had no significant influence on the cleavage kinetics, we prepared Boc-Phe-Glu($OC_{12}H_{25}$)-Ala-NH_2 (**4d**; Table 1) as an additional model peptide. Peptide **4d** contains a dodecyl glutamate moiety and mimics peptides derived from endogenous proteins bearing lipid esters in this position (such as **C** in Scheme 1). Peptide **4d** was prepared from **4a** and dodecan-1-ol by transesterification with the aid of sodium cyanide as catalyst.[32] Alternative attempted direct esterification of **9a** by the standard methods of Steglich[33] or Mitsunobu[34] were not successful in this case. Compound **4d** was subjected to the cleavage conditions and showed transesterification and cleavage rates similar to those of the model peptides **4a** and **4b** (data not shown).

We plan to apply the conditions used for the cleavage of the model peptides to human skin proteins, with subsequent ESI-MS screening for the occurrence of peptides containing N-terminal pyroglutamate residues. Database alignment of the observed masses with skin protein peptide sequences should allow the identities of the modified proteins and the altered glutamate residues to be determined. A frequently encountered phenomenon in the analysis of peptides by mass spectrometry is the occurrence of cleavage products on the carboxy sides of glutamic and aspartic acid residues, observed when multiply charged protein ions fragment into cleavage products with C-terminal anhydride residues during ESI-MS measurements.[26] This reaction does not interfere with the strategy outlined in this manuscript. On the other hand, it has to be argued that N-terminal pyroglutamate-containing peptides could be formed during preparation, purification,[27] or mass spectrometric analysis[28] of peptides with N-terminal glutamine residues, or by their enzymatic conversion.[29–31] To investigate the extent to which terminal pyroglutamate residues from N-terminal glutamine-containing peptides were formed under the reaction conditions developed by us for site-specific cleavage, we investigated peptide **4e** (H-Gln-Phe-Phe-Gly-Leu-Met-NH_2) as a model substance. After 24 h treatment under the cleavage conditions, about 40% of this peptide was recovered, and the corresponding derivative with the N-terminal pyroglutamate residue was detected in amounts of about 25%. This indicates that sequence alignment of diagnostic cleavage products derived from skin samples should be conducted with care to exclude false positive sequences originating from peptides with N-terminal glutamine residues. For mechanistic reasons, internal glutamines do not interfere with the method; this was also experimentally demonstrated with peptide **4c**, with which no products due to cleavage at this position could be detected.

As shown for peptide **4c**, a case in which a dehydroalanine-containing cleavage product was also obtained, methoxide treatment can lead to amino acid modifications that have to be taken into account during MS analysis. Other possible side reactions are the deamidation of asparagine residues, the elimination of methanesulfenic acid from oxidized methionine or the deglycosylation of *O*-glycosylated glycopeptides.

In this work we have investigated the cleavage of glutamic acid ester-containing peptides as a tool for the determination of residues modified in this way post-translation. This method appears to be especially attractive, since methoxide should be able to penetrate into the rigid structure of the cornified envelope and might facilitate peptide cleavage within the protein network. This highly crosslinked protein aggregate is not otherwise easily degradable, because proteases can only operate at the periphery of this network. In addition, products of site-specific cleavage are easier to detect than labelling products such as the methyl esters transiently formed during the cleavage conditions. We have developed conditions that have proved successfully applicable to model peptides and are currently applying this strategy, together with other techniques, for the determination of lipid-modified human skin proteins by mass spectrometry.

Experimental Section

General: Glassware was flame-dried, and reactions were carried out under argon. The employed amino acid derivatives **1** and **2** and peptides **4c, 4e** and **8a** are available from Bachem (Bubendorf, Switzerland). Peptide **4e** corresponds to residues (6–11) of substance P. Chemical reagents were purchased from Fluka (Taufkirchen, Germany). Peptide derivatives were separated by reversed-phase HPLC on a Luna RP-18(2) column (250×2 mm; 5 μm, Phenomenex, Aschaffenburg, Germany) connected to a SMART System from Amersham Pharmacia (Freiburg, Germany). For cleavage of peptides, three different solvent systems were used (compare Table 1). For resolution of compounds **4a**, **4b** and **4d**, for example, solvent system I—consisting of HPLC grade water and methanol in a 50:50 (vol/vol) ratio as solvent A, and methanol as solvent B—was used. The column was equilibrated with solvent A at ambient temperature, and the peptides were eluted with a linear gradient of 0–100% solvent B over 10 minutes at a flow rate of 200 μL min^{-1}. Detection was performed at 210 and 260 nm. For peptide **4c** and derivatives, a linear gradient from 5–95% acetonitrile in water and 0.1% trifluoroacetic acid (=solvent system II) over 60 minutes was used. The cleavage of peptide **4e** was analysed by HPLC by use of solvent system I with addition of 0.1% trifluoroacetic acid (=solvent system III).

Melting points were determined on a Büchi SMP 20 capillary melting point apparatus and are uncorrected. Optical rotations were measured at 25 °C on a Perkin–Elmer P341 polarimeter. ^{1}H NMR spectra: Bruker AM 400 (400 MHz) and Bruker AM 300 (300 MHz) instruments, solvent as internal standard ($CDCl_3$: $\delta_H = 7.24$ ppm). ^{13}C NMR spectra: Bruker AM 400 (100 MHz) and Bruker AM 300 (75 MHz) instruments, solvent as internal standard ($CDCl_3$: $\delta_C = 77.0$ ppm). ^{13}C NMR spectra were recorded in broadband decoupled mode, and multiplicities were determined by use of a DEPT pulse sequence.

Mass spectra were recorded in positive ion mode on a Q-TOF 2 mass spectrometer (Micromass, Manchester, UK) fitted with a nanospray source. Analytes were dissolved in acetonitrile/water 2:1 (*v*/*v*) and were injected into the mass spectrometer through glass capillaries (long type; Protana, Odense, Denmark) with use of a capillary voltage of 1000 V and a cone voltage of 50 V. Instrument calibration was carried out with a mixture of sodium iodide and cesium iodide dissolved in aqueous propan-2-ol (50%).

Column chromatography: silica gel 60 (E. Merck, Darmstadt, Germany), thin layer chromatography: (silica gel plates 60, thickness 0.25 mm, E. Merck, Darmstadt, Germany). Elemental analyses were performed in the microanalytical department of the Kekulé-Institut für Organische Chemie und Biochemie, Bonn, Germany.

***N-tert*-Butoxycarbonyl deprotection**: The *N*-terminal Boc-protected peptide component (1 mmol) was mixed at 0 °C with trifluoroacetic acid (2 mL). After the mixture had been stirred for 2 h at room temperature, the trifluoroacetic acid was removed under reduced pressure, and the remaining residue was treated with diethyl ether and dried. The material was used in the next step without further purification.

Peptide coupling: Peptides were synthesized by a modification of the DCC/HOBt method[21,22] with the use of EDC/HOBt[23] (EDC = *N*′-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide hydrochloride). Since traces of dicyclohexylurea could not be completely removed when working with DCC, we used the EDC/HOBt method in this work.

Et_3N (1 equiv) and HOBt (0.1 equiv) were added to a solution of *N*-Boc-protected amino acid and the C-terminal-protected component in water (2.5 mL, 1 mmol). The solution was cooled to 0°, and EDC (1 equiv) was added. The mixture was stirred for 2 h at ambient temperature and was then extracted with water/ethyl acetate. Depending on the solubility of the target compound, either the organic layer was separated, dried (Na_2SO_4) and evaporated under reduced pressure, or the aqueous phase was evaporated. The remaining residue was purified by chromatography on silica gel with ethyl acetate/methanol gradients as solvent.

Affinity cleavage conditions: The peptide derivative (10 mg) was dissolved in a solution of sodium methoxide in abs. methanol (0.25 M, 6 mL). At different time points, aliquots (100 μL) were taken out of the reaction vessel by syringe and neutralised with acetic acid solution (0.25 M, 100 μL). This solution was analysed by HPLC. It is important to note that non-esterified glutamate residues (but not aspartate residues) have been transformed into the methyl esters in detectable amounts in peptides isolated from human *stratum corneum* by use of methanol-containing solvent mixtures. Therefore, care has to be taken that the use of methanol as solvent be avoided during sample preparation.

For HPLC quantification, a calibration curve was established by injection of known amounts of peptide **4a** and the corresponding methyl ester **5a**. The curves for the peak areas at 260 nm were linear over the ranges from 0–150 nmol for **4a** and 0–20 nmol for **5a.** The cleavage product **8a** was quantified by use of the same peak height at 260 nm in the range from 0–200 nmol. Peptides **4b** (peak height at 260 nm, range 0–150 nmol), **5b** (peak height at 210 nm, range 0–12 nmol), **8b** (peak height at 210 nm, range 0–80 nmol) and **9b** (peak height at 210 nm, range 0–50 nmol) were quantified in the same way. For the detection of **9b**, TFA (0.1%) had to be added to the HPLC solvents.

Analytical data

***N-tert*-Butoxycarbonyl-(*O*-benzyl-L-glutamyl)-L-alanylamide (3)**: Chromatography (silica gel, ethyl acetate) gave **3** (950.7 mg, 78.8% yield); m.p. 136 °C; ^{1}H NMR ($CDCl_3$): $\delta = 7.27$ (m, 5H; aryl), 5.05 (s, 2H; $BnCH_2$), 4.41 (m, 1H; CH), 4.06 (m, 1H; CH), 2.43 (m, 2H; Glu-CH_2), 2.07 (m, 1H; Glu-CH_2), 1.90 (m, 1H; Glu-CH_2), 1.35 (s, 9H; $(CH_3)_3$), 1.31 ppm (d, 3H, $J = 7.0$ Hz; Ala-CH_3); four NH protons were detected when measured in [D_5]pyridine: $\delta = 6.82$ (d, $J = 6.6$ Hz, 1H), 6.49 (br m, 1H), 5.61 (br m, 1H), 5.42 ppm (d, $J = 4.5$ Hz, 1H); ^{13}C NMR ($CDCl_3$): $\delta = 174.6$, 173.3, 171.6, 156.0, 135.6, 128.6, 128.4,

128.3, 80.6, 66.7, 54.5, 48.6, 30.6, 28.3, 28.3, 17.8 ppm; MS (ESI): *m/z* calcd for $C_{20}H_{29}N_3NaO_6$: 430.20 $[M+Na]^+$; found 430.11.

***N-tert*-Butoxycarbonyl-L-phenylalaninyl-(*O*-benzyl-L-glutamyl)-L-alanylamide (4a)**: Chromatography (silica gel, cyclohexane/ethyl acetate 1:1 to cyclohexane/ethyl acetate 1:10) afforded **4a** (654.1 mg) in 76.6% yield. M.p. 177–8 °C; ^{1}H NMR ($CDCl_3/CD_3OD$ 1:1): δ = 7.13–7.32 (m, 10H; Bn), 5.01 (s, 2H; CH_2), 4.23–4.31 (m, 3H; Glu-CH, Ala-CH, Phe-CH), 3.06 (dd, J = 13.9, 5.5 Hz, 1H; Glu-CH_2), 2.86 (dd, J = 14, 8 Hz, 1H; Glu-CH_2), 2.11 (m, 1H; Glu-CH_2), 1.93 (m, 1H; Glu-CH_2), 1.36–1.33 ppm (d, J = 7.2 Hz, 12H; Ala-CH_3, $(CH_3)_3$); ^{13}C NMR ($CDCl_3/CD_3OD$ 1:1): δ = 177.2, 174.7, 174.6, 172.7, 157.8, 137.9, 137.1, 130.5, 129.8, 129.85, 129.7, 129.5, 128.2, 81.6, 67.9, 57.6, 54.4, 50.2, 38.9, 34.9, 31.5, 29.2, 18.6 ppm; MS (ESI): *m/z* calcd for $C_{29}H_{38}N_4NaO_7$: 577.27 $[M+Na]^+$; found 577.34; elemental analysis calcd (%) for $C_{29}H_{38}O_7N_4$: C 62.8, H 6.91, N 10.1; found C 62.54, H 6.59, N 10.15; HPLC retention time = 13.8 min (solvent system I).

***N-tert*-Butoxycarbonyl-L-valinyl-(*O*-benzyl-L-glutamyl)-L-alanylamide (4b)**: Chromatography (silica gel, ethyl acetate) afforded **4b** (1123 mg, 64.2%); m.p. 192 °C; ^{1}H NMR ($CDCl_3/CD_3OD$ 1:1): δ = 7.24 (m, 5H; Bn), 5.02 (s, 2H; CH_2), 4.63 (t, J = 6.0 Hz, 1H; Glu-CH), 4.27 (q, J = 7.2 Hz, 1H; Ala-CH), 3.77 (d, J = 5.9 Hz, 1H; Val-CH), 2.88 (dd, J = 16.8, 6.7 Hz, 1H; Glu-CH_2), 2.79 (dd, J = 16.8, 5.8 Hz, 1H; Glu-CH_2), 1.96 (m, 3H; Val-C$H$$(CH_3)_2$ and Glu-CH_2), 1.37 (s, 9H; $(CH_3)_3$), 1.30 (d, J = 7.2 Hz, 3H; Ala-CH_3), 0.86 (d, J = 6.8 Hz, 3H; Val-CH_3), 0.82 ppm (d, 3H, J = 6.8 Hz; Val-CH_3); ^{13}C NMR ($CDCl_3/CD_3OD$ 1:1): δ = 177.2, 174.5, 172.5, 171.8, 158.1, 136.7, 129.9, 129.7, 129.5, 81.7, 68.3, 61.9, 51.2, 50.4, 49.9, 36.6, 30.9, 29.1, 20.2, 19.0, 18.6 ppm; MS (ESI): *m/z* calcd for $C_{25}H_{38}N_4NaO_7$: 529.27 $[M+Na]^+$; found 529.35; elemental analysis calcd (%) for $C_{25}H_{38}O_7N_4$: C 59.27, H 7.56, N 11.06; found C 59.29, H 7.68, N 10.89; HPLC retention time = 12.8 min (solvent system I).

***N-tert*-Butoxycarbonyl-L-phenylalanyl-(*O*-dodecyl-L-glutamyl)-L-alanylamide (4d)**: Transesterification of **4a** to **4d** was carried out by the method of Mori et al.[32]

Compound **4a** (100 mg) and NaCN (35 mg) were dissolved in DMSO (20 mL). Dodecan-1-ol (200 mg) was added, and stirring was continued for 48 h at room temperature. The reaction mixture was diluted with ethyl acetate and washed with sat. $NaHCO_3$ and then with water. The organic layer was dried over Na_2SO_4 and evaporated. Analysis of peak forms in HPLC point to up to 50% racemization under the reaction conditions.

Chromatography (silica gel, ethyl acetate; R_f = 0.34) afforded **4d** (40 mg, 35.0%) as a waxy solid. ^{1}H NMR ($CDCl_3$): δ = 7.11–7.30 (m, 5H; Bn), 5.40 (br, 1H; NH), 5.34 (br, 1H; NH), 5.28 (br, 2H; NH), 5.00 (d, J = 7.3 Hz, 1H; NH), 4.91 (s, 2H; CH_2), 3.92–4.16 (m, 3H; Glu-CH, Ala-CH, Phe-CH), 3.08 (dd, J = 14.1, 4.9 Hz, 1H; Glu-CH_2), 2.87 (dd, J = 14.1, 8.4 Hz, 1H; Glu-CH_2), 2.41 (m, 2H; alkyl-CH_2), 2.28 (m, 2H; alkyl-CH_2), 2.05 (m, 1H; Glu-CH_2), 1.95 (m, 1H; Glu-CH_2), 1.86 (m, 2H; alkyl-CH_2), 1.31–1.34 (m, 12H; Ala-CH_3, $(CH_3)_3$), 1.19 (m, 16H; alkyl-CH_2), 0.81 ppm (t, J = 6.8 Hz, 3H; alkyl-CH_3); ^{13}C NMR ($CDCl_3$): δ = 175.1, 173.4, 171.7, 170.6, 154.4, 135.6, 129.2, 129.0, 127.5, 81.3, 65.6, 57.2, 49.2, 49.1, 33.9, 31.9, 29.3–29.7, 28.5, 28.2–28.3, 26.0, 25.5, 24.9, 22.8, 17.8, 14.1 ppm; MS (ESI): *m/z* calcd for $C_{34}H_{56}N_4NaO_7$: 655.40 $[M+Na]^+$; found 655.40; HPLC retention time = 17.2 min (solvent system I).

Cleavage of H-Gln-Phe-Phe-Gly-Leu-Met-NH_2 (4e): Peptide **4e** turned out to be stable for 4 h under the cleavage conditions (retention time 10.81 min, solvent system III), but after 24 h only 40 mol% were detectable. About 25 mol% of the *N*-terminal pyroglutamate-containing product could be detected (retention times 12.12 and 12.87 min; solvent system III).

***N-tert*-Butoxycarbonyl-L-phenylalanyl-(*O*-methyl-L-glutamyl)-L-alanylamide (5a)**: Transesterification of **4a** to **5a** was carried out by the method of Giannis et al.[25] Chromatography (silica gel, ethyl acetate/methanol 10:1) gave **5a** (51.2 mg, 76.0% yield). M.p. 174 °C; ^{1}H NMR (CD_3OD): δ = 7.10–7.19 (m, 5H; Ar), 4.18–4.28 (m, 3H; Phe-CH, Glu-CH, Ala-CH), 3.02 (dd, J = 13.9, 5.3 Hz, 1H; Bn-CH_2), 3.57 (s, 3H; Glu-OMe), 2.76 (dd, J = 13.9, 9.2 Hz, 1H; Bn-CH_2), 2.32 (m, 2H; Glu-CH_2), 2.03 (m, 1H; Glu-CH_2), 1.86 (m, 1H; Glu-CH_2), 1.27–1.29 ppm (m, 12H; Boc-$(CH_3)_3$, Ala-CH_3); ^{13}C NMR (CD_3OD): δ = 176.0, 173.7, 173.3, 171.6, 155.4, 133.0, 128.9, 128.2, 126.3, 79.4, 56.2, 52.7, 50.8, 48.8, 37.3 (CH_2), 29.5 (CH_2), 27.2, 26.6 (CH_2) 16.8 ppm; MS (ESI): *m/z* calcd for $C_{23}H_{34}N_4NaO_7$: 501.23 $[M+Na]^+$; found 501.20; HPLC retention time = 11.9 min (solvent system I).

***N-tert*-Butoxycarbonyl-L-valinyl-(*O*-methyl-L-glutamyl)-L-alanylamide (5b)**: Chromatography (silica gel, ethyl acetate/methanol 10:1) gave **5b** (51.2 mg, 12.0% yield). M.p. 207 °C; ^{1}H NMR ($CDCl_3/CD_3OD$ 1:1): δ = 4.22 (q, J = 7.2 Hz, 1H; Ala-CH), 3.71 (d, J = 6.2 Hz, 1H; Val-CH), 3.56 (s, 3H; OCH_3), 2.33 (m, 1H; Val-C$H$$(CH_3)_2$), 2.02 (m, 2H; Glu-C$H_2$), 1.88 (m, 2H; Glu-C$H_2$), 1.33 (s, 9H; $(CH_3)_3$), 1.27 (d, J = 7.3 Hz, 3H; Ala-CH_3), 0.84 (d, J = 6.8 Hz, 3H; Val-CH_3), 0.81 ppm (d, J = 6.8 Hz, 3H; Val-CH_3); ^{13}C NMR ($CDCl_3/CD_3OD$ 1:1): δ = 177.3, 175.5, 175.0, 172.9, 158.3, 81.4, 62.2, 54.5, 52.9, 50.3, 31.8, 31.3, 27.7, 20.1 19.0, 18.6 ppm; MS (ESI): *m/z* calcd for $C_{19}H_{34}N_4NaO_7$: 453.24 $[M+Na]^+$; found 453.27; HPLC retention time = 10.9 min (solvent system I).

Pyroglutamylalaninamide (7a/b) obtained under affinity cleavage conditions: Chromatography (silica gel, ethyl acetate/methanol 1:2) afforded **7a/b** as a colourless oil (33.8 mg, 17.3% yield); mixture of two stereoisomers, ratio 1:1. ^{1}H NMR ($CDCl_3/CD_3OD$ 1:1): δ = 4.27 (2 × q, J = 7.2 Hz, 1H; Ala-CH), 4.13 (m, 1H; Pyroglu-CH), 2.34 (m, 1H; CH_2), 2.21 (m, 1H; Glu-CH_2), 2.00 (m, 1H; CH_2), 1.29 ppm (d, J = 7.2 Hz, 3H; Ala-CH_3); ^{13}C NMR ($CDCl_3/CD_3OD$ 1:1): δ = 181.6, 181.5, 177.4, 174.6, 174.6, 58.0, 50.1, 30.5, 30.5, 26.5, 26.5, 18.3 ppm; MS (ESI): *m/z* calcd for $C_8H_{13}N_3NaO_3$: 222.06 $[M+Na]^+$; found 222.05; HPLC retention time = 3.9 min.

Enantiomeric pure sample of (7a/b), L form: Chromatography (silica gel, acetone/water 1:1) afforded **7a/b** (242.7 mg, 81.3%); m.p. 163 °C; ^{1}H NMR ($CDCl_3/CD_3OD$ 1:1): δ = 4.37 (q, J = 7.2 Hz, 1H; Ala-CH), 4.21 (dd, J = 8.4, 5.0 Hz, 1H; Pyroglu-CH), 2.42 (m, 1H; CH_2), 2.28 (m, 2H; CH_2), 2.11 (m, 1H; CH_2), 1.37 ppm (d, 3H, J = 7.2 Hz; Ala-CH_3); ^{13}C NMR ($CDCl_3/CD_3OD$ 1:1): δ = 181.6, 177.4, 174.6, 58.0, 50.1, 30.5, 26.5, 18.3 ppm; MS (ESI): *m/z* calcd for $C_8H_{13}N_3NaO_3$: 222.06 $[M+Na]^+$; found 222.05; HPLC retention time = 3.3 min (solvent system I).

Pyroglu-Val-Glu-Asp-Gln-Lys-Glu-Glu-OH (7c): MS (ESI): *m/z* calcd for $C_{40}H_{63}N_{10}O_{19}$: 987.42 $[M+H]^+$; found 987.4; HPLC retention time = 10.9 min (solvent system II).

***N-tert*-Butoxycarbonyl-L-phenylalanine methyl ester (8a)**: Compound **8a** is commercially available from Bachem (Bubendorf, Switzerland); HPLC retention time = 13.3 min (solvent system I).

***N-tert*-Butoxycarbonyl-L-valine methyl ester (8b)**: Chromatography (silica gel, ethyl acetate/methanol 1:1) gave **8b** as a colourless oil (27.7 mg, 12.1% yield). ^{1}H NMR ($CDCl_3$): δ = 4.94 (br m, 1H; NH), 4.15 (dd, J = 8 Hz, 4.4 Hz, 1H; Val-CH), 3.66 (s, 3H; OCH_3), 2.04 (m, 1H; C$H$$(CH_3)_2$), 1.38 (s, 9H; $(CH_3)_3$), 0.89 (d, J = 6.8 Hz, 3H; Val-CH_3), 0.82 ppm (d, J = 6.9 Hz, 3H; Val-CH_3); ^{13}C NMR ($CDCl_3$): δ = 172.9, 155.7, 79.8, 58.6, 52.0, 31.9, 28.3, 19.0, 17.7 ppm; MS (ESI): *m/z*

calcd for $C_{11}H_{21}NNaO_4$: 254.15 $[M+Na]^+$; found 254.09; HPLC retention time = 12.6 min (solvent system I).

***N-tert*-Butoxycarbonyl-L-phenylalaninyl-L-glutamyl-L-alanylamide (9a)**: Compound **9a** was obtained on cleavage of **4a**. MS (ESI): *m/z* calcd for $C_{22}H_{32}N_4O_7Na$: 487.22 $[M+Na]^+$; found 487.21; HPLC retention time = 13.9 min (solvent system I).

***N-tert*-Butoxycarbonyl-L-valinyl-L-glutamyl-L-alanylamide (9b)**: Compound **4b** (200 mg, 0.395 mmol) was dissolved in argon-saturated methanol (50 mL), and palladium on charcoal (10%, 50 mg) was added. The solution was stirred for 18 h under a hydrogen atmosphere and was then filtered and evaporated under reduced pressure.

Chromatography (silica gel, dichloromethane/methanol 3:1 + 0.1% HOAc) afforded **9b** (132,1 mg, 89.3% yield); m.p. 184 °C; ^{1}H NMR (CD_3OD): $\delta = 4.28$ (dd, $J = 8.2$, 5.3 Hz, 1H; Glu-CH), 4.23 (q, $J = 7.2$ Hz, 1H; Ala-CH), 3.74 (d, $J = 6.6$ Hz, 1H; Val-CH), 2.33 (m, 1H; Val-C*H*$(CH_3)_2$)), 1.87–2.02 (m, 4H; Glu-CH_2), 1.36 (s, 9H; $(CH_3)_3$), 1.28 (d, $J = 7.3$ Hz, 3H; Ala-CH_3), 0.86 (d, $J = 6.8$ Hz, 3H; Val-CH_3), 0.82 ppm (d, $J = 6.8$ Hz, 3H; Val-CH_3); ^{13}C NMR (CD_3OD): $\delta = 177.5$, 176.8, 175.5, 173.2, 158.3, 80.9, 62.1, 54.3, 50.3, 49.0, 31.1, 30.7, 28.7, 19.7, 18.6, 18.2 ppm; MS (ESI): *m/z* calcd for $C_{32}H_{44}N_4NaO_7$: 439.23 $[M+Na]^+$; found 439.16; HPLC retention time = 8.7 min (solvent system III).

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