Identification and Quantitation of New Glutamic Acid Derivatives in Soy Sauce by UPLC/MS/MS

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Glutamic acid is an abundant amino acid that lends a characteristic umami taste to foods. In fermented foods, glutamic acid can be found as a free amino acid formed by proteolysis or as a non-proteolytic derivative formed by microorganisms. The aim of the present study was to identify different structures of glutamic acid derivatives in a typical fermented protein-based food product, soy sauce. An acidic fraction was prepared with anion-exchange solid-phase extraction (SPE) and analyzed by UPLC/MS/MS and UPLC/TOF-MS. α-Glutamyl, γ-glutamyl, and pyroglutamyl dipeptides, as well as lactoyl amino acids, were identified in the acidic fraction of soy sauce. They were chemically synthesized for confirmation of their occurrence and quantified in the selected reaction monitoring (SRM) mode. Pyroglutamyl dipeptides accounted for 770 mg/kg of soy sauce, followed by lactoyl amino acids (135 mg/kg) and γ-glutamyl dipeptides (70 mg/kg). In addition, *N*-succinoylglutamic acid was identified for the first time in food as a minor compound in soy sauce (5 mg/kg).

Introduction. – In the 1970s, Japanese researchers studied the small peptides in soy sauce [1–3]. Using gel filtration, ion-exchange chromatography, and paper chromatography, coupled with amino acid analysis, they identified many acidic dipeptides (Glu-Glu, Asp-Glu, Glu-Ala, *etc.*) considered to contribute to the *umami* taste of soy sauce [1]. More recent studies [4–7] questioned the contribution of small peptides, and revealed that amino acids and sodium salts were responsible for this umami taste. *Toelstede* and *Hofmann* [8][9] identified a series of α-glutamyl and γ-glutamyl dipeptides in cheeses, and *Toelstede et al.* [9] identified many γ-glutamyl dipeptides in a study on the maturation of Gouda cheese. The food industry is actively seeking substitutes for monosodium glutamate that either occur naturally in foods or plants, or can be produced artificially [10].

At *Firmenich*, *Frerot* and *Escher* [11] studied the contribution of synthetic glutamyl tripeptides and lactoyl amino acids to the taste of cheese-like solutions. Chemically synthesized succinoyl amino acids such as *N*-succinyl glutamate were also found to be taste-active [12], but were never reported to occur in foods.

In this work, we analyzed a commercial and widely distributed soy sauce, focusing on glutamic acid derivatives, in order to show the natural occurrence of many amino acid derivatives.

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Results and Discussion. – The study was performed in four steps: preparation of an acidic fraction of soy sauce, identification of the peptides and amino acid derivatives, synthesis of unavailable reference products for confirmation, and calibration and quantification of identified compounds.

For the preparation of an acidic fraction of soy sauce, solid-phase extraction (SPE) was performed using a mixed-mode polymeric reversed-phase anion-exchanger (*Oasis MAX*). These SPE cartridges were used for microbial natural-product detection and they showed consistency and high loading capacity [13]. Although the '*Less Salt*' soy sauce contained less sodium chloride, it had to be diluted 167 × in H_2O before loading. After the loading, rinsing, and elution, the acidic fraction was obtained as a 5% HCOOH solution that was 20-fold diluted in relation to the genuine soy sauce. It was used as such for both qualitative and quantitative analyses.

The acidic fraction of soy sauce was analyzed by ultra-performance liquid chromatography/mass spectrometry (UPLC/MS). As current UPLC columns can sustain pure aqueous solvent, a good separation was obtained even for the most polar compounds. The first analyses were performed on ion-trap and time-of-flight (TOF) instruments because of their high scanning speed, which was suitable for UPLC. *Fig. 1* shows the negative full-scan chromatogram obtained by electrospray ionization (ESI). The first identification of peaks was achieved from the positive- and negative-ion mode MS/MS spectra obtained with the ion-trap mass spectrometer, in addition to the molecular formula calculated from TOF data. In *Table 1*, the identities of the peaks and the spectral data that led to structural determination, as recommended by the *International Organization of the Flavor Industry* (*IOFI*) [14], were compiled. These compounds belong to four families of amino acid derivatives, as shown in *Fig. 2*. Their identification was confirmed by comparison with the spectral and chromatographic data of reference products synthesized by classic methods. Pyroglutamyl dipeptides

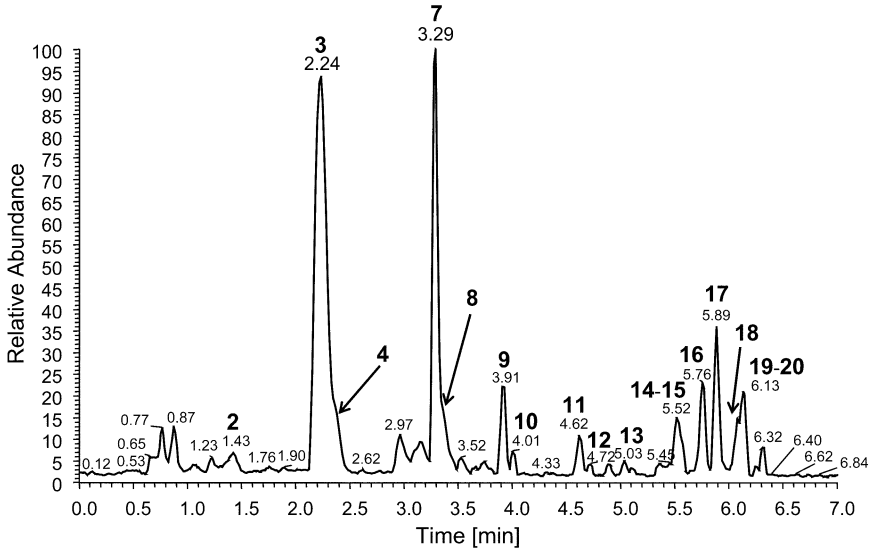

Fig. 1. *Negative ESI full-scan trace of soy-sauce acidic extract*

Table 1. *Compounds Identified in the Acidic Fraction of Soy Sauce*

	Reaction time [min]	Nominal mass[a])	MS/MS fragments[a])		Exact mass[b])		Calc. molecular formula	Compound[c])
			Positive-ion mode	Negative-ion mode	$[M+H]^+$	$[M-H]^-$		
1	1.17	276				275.0856	$C_{10}H_{16}N_2O_7$	Glu-Glu[d])
2	1.43	276	148, 130, 102, 84	257, 239, 146, 128	277.10511	275.0924	$C_{10}H_{16}N_2O_7$	γGlu-Glu
3	2.24	192		173, 111		191.01846	$C_6H_8O_7$	Citric acid
4	2.33	244	227, 199, 134	225, 199, 128, 127		243.06071	$C_9H_{12}N_2O_6$	pGlu-Asp
5	2.6	189				188.05579	$C_7H_{11}NO_5$	N-acetyl-Glu[d])
6	3.27	247				246.06344	$C_9H_{13}NO_7$	Suc-Glu[d])
7	3.3	258	241, 148, 130	239, 128		257.08051	$C_{10}H_{14}N_2O_6$[e])	pGlu-Glu
8	3.33	219		200, 128, 89		218.07011	$C_8H_{13}NO_6$	Lac-Glu
9	3.93	226	209, 116	181		225.091	$C_{10}H_{14}N_2O_4$[e])	pGlu-Pro
10	3.98	165	149, 120		166.08778		$C_9H_{11}NO_2$	Phe
11	4.62	228	211, 183, 118	183		227.10294	$C_{10}H_{16}N_2O_4$	pGlu-Val[f])
12	4.72	260	244, 132, 86	241, 128	261.14619	259.1287	$C_{11}H_{20}N_2O_5$	γGlu-Ile[f])
13	5.03	260	244, 132, 87	241, 128	261.14506		$C_{11}H_{20}N_2O_5$	γGlu-Leu
14	5.52	294	278, 166, 120	275, 128	295.13213	293.1119	$C_{14}H_{18}N_2O_5$	γGlu-Phe
15	5.57	189	172, 144	144, 116, 100			$C_8H_{15}NO_4$	Lac-Val
16	5.76	242	225, 197, 132	197		241.12115	$C_{11}H_{18}N_2O_4$	pGlu-Ile
17	5.89	242	225, 197, 133	197		241.1239	$C_{11}H_{18}N_2O_4$[e])	pGlu-Leu
18	6.07	276	259, 231, 166, 120	231, 147, 127, 109		275.10159	$C_{14}H_{16}N_2O_4$	pGlu-Phe
19	6.09	203	186, 158	158, 130, 114				Lac-Ile[f])
20	6.14	203	186, 158	158, 130, 114		202.10984	$C_9H_{17}NO_4$[e])	Lac-Leu

[a]) Determined on an ion-trap instrument. The relative intensities of MS/MS fragment-ion peaks in soy sauce within ±20% of those of the synthetic products. [b]) Determined on a time-of-flight (TOF) MS instrument. All calculated molecular formulae within ±10 ppm of the determined exact masses. [c]) At least four points of identification obtained according to *IOFI* guidelines [14]. [d]) SRM recorded on a triple quadrupole MS instrument. Ratio Transition 1 Transition 2 within ±20% of that of the synthetic compounds. [e]) Deviation from measured exact mass <25 ppm. [f]) Tentative identification based on MS data. Products **11**, **12**, and **19** were not synthesized.

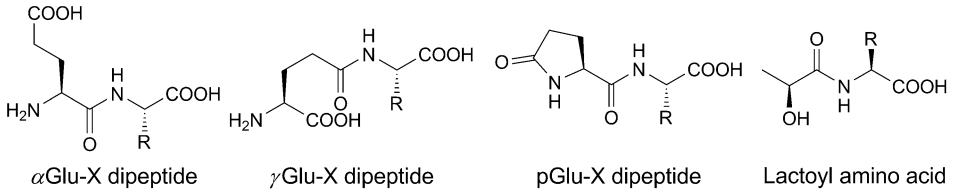

Fig. 2. *Dipeptides and amino acid derivatives found in soy-sauce acidic extract*

(*Fig. 2*) were the major compounds. In 1970, pGlu-Glu (**7**) and pGlu-Pro (**9**) were identified in the edible mushroom *Agaricus campestris* [15]. pGlu-Gly and pGlu-Gln were recently described in soy sauce [6]. We could not identify these two compounds by the full-scan experiments (*Fig. 1*). γ-Glutamyl dipeptides are ubiquitous compounds in foods [8][9][16][17]. Lactoyl amino acids were first discovered by *Firmenich* during the analysis of Parmesan [18] and patented for their taste properties [19]. A thorough study of Parmesan and other Italian cheeses revealed that pyroglutamyl dipeptides, lactyl amino acids, and γ-glutamyl dipeptides accumulated during ripening, probably because they could no longer be recognized by hydrolytic enzymes [20]. While the four families of amino acid derivatives identified in this work were already known, the most polar compounds had never been previously described. In this work, pGlu-Asp (**4**), pGlu-Val (**11**), and Lac-Glu (**8**) were unambiguously identified in a food product for the first time.

Other taste-active glutamic acid derivatives were already described in foods: αGlu-Glu (**1**) in cheeses [8][9], and *N*-acetylglutamic acid (**5**) in soybean seeds [21] and in fermented tuna [22]. The two products **1** and **5**, as well as *N*-succinoylglutamic acid (**6**) [12], could not be identified by the first full-scan experiments using the ion-trap MS, but were detected by the TOF-MS. Their occurrence in soy sauce was firmly established by determining the MS transition ratios in the selected reaction monitoring mode (SRM), using the triple quadrupole mass spectrometer. The ratio of the peak area corresponding to two transitions was determined at different concentrations for the synthetic products. This ratio was subsequently compared with that actually found in soy sauce to ensure correct identification of the compounds, following the *IOFI* guidelines [14]. Thus, the presence of αGlu-Glu (**1**), Suc-Glu (**6**), Ac-Glu (**5**), and Lac-Glu **8** (*Fig. 3*) in soy sauce could be evidenced by SRM recordings in both the positive- and the negative-ionization modes, and it exceeded *IOFI* recommendations.

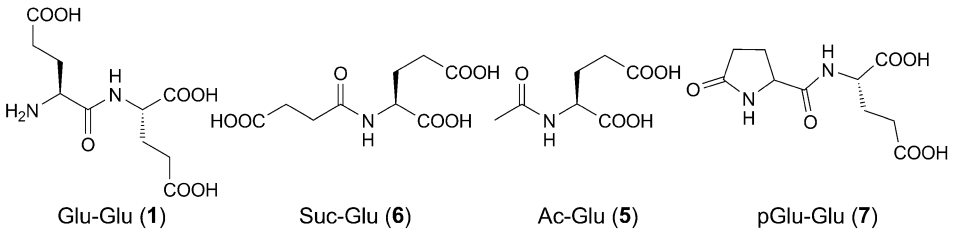

Fig. 3. *Additional dipeptides and amino acid derivatives in-soy sauce acidic extract detected by UPLC/MS/MS in the SRM mode*

The concentrations of the identified compounds were determined by UPLC/MS in the SRM mode. The external calibration curves were obtained from the synthetic products (0.05–50 ppm) using two mass transitions. As there was no anticipated sensitivity issue, the MS conditions were not fully optimized, and the same collision energy was used for all compounds. A total of 16 compounds were quantified in an SPE *Oasis MAX* extract of soy sauce. The recoveries during the SPE sample-preparation step were not investigated, but were described as nearly quantitative for weak acids [23]. For most of these compounds, the quantitative results obtained from positive- and negative-ion ESI SRM experiments were in good agreement, thereby minimizing the possibility of biased results due to ion-suppression effects. In *Table 2*, the results expressed as the concentration in parts per million (mg/l) in the original soy sauce are collected. The quantified pyroglutamyl dipeptides accounted for a total of 770 ppm, lactoyl amino acids for 135 ppm, and γ-glutamyl dipeptides for only 70 ppm. Suc-Glu (**6**) accounted for less than 10 ppm.

Table 2. *Concentration of Acidic Amino Acid Derivatives in Soy Sauce*

Product	Concentration in soy sauce [mg/l]	
	Negative-ion mode[a])	Positive-ion mode[b])
Glu-Glu (**1**)	5.0	6.6
γGlu-Glu (**2**)	18.4	25.2
pGlu-Asp (**4**)	207.5	245.7
N-acetyl-Glu (**5**)	8.1	10.3
Suc-Glu (**6**)	5.4	8.0
pGlu-Glu (**7**)	344.8	339.6
Lac-Glu (**8**)	82.1	85.5
pGlu-Pro (**9**)	121.8	144.3
γGlu-Ile[c]) (**12**)	11.3	13.4
γGlu-Leu (**13**)	11.6	14.0
γGlu-Phe (**14**)	30.3	31.5
Lac-Val (**15**)	40.0	n.d.[d])
pGlu-Ile[e]) (**16**)	48.0	53.6
pGlu-Leu (**17**)	46.3	44.1
pGlu-Phe (**18**)	1.4	3.0
Lac-Leu (**20**)	12.5	20.7

[a]) Quantified from negative-ion ESI-MS. [b]) Quantified from positive-ion ESI-MS. [c]) Same calibration curve as for γGlu-Leu used. [d]) n.d., Not determined. [e]) Same calibration curve as for pGlu-Leu used.

During fermentation of soy and wheat proteins, the so-called *Koji* fermentation, α-glutamyl dipeptides are formed by proteolytic enzymes present in the *Aspergillus* microorganism [5], whereas γ-glutamyl dipeptides are formed by γ-glutamyl transferases [8]. Pyroglutamyl dipeptides are assumed to form from the corresponding α-glutamyl dipeptides (α-Glu-Aaa) or from α-glutaminyl dipeptides (α-Gln-Aaa). No enzyme is needed, since heating was shown to be sufficient to perform the cyclization reaction [24]. The enzymatic coupling of pyroglutamic acid to a free amino acid was also proposed [15]. The formation of lactyl amino acids in Parmesan cheese was briefly discussed, and a coupling reaction between L-lactic acid and free L-amino acid was

proposed [20]. It should be mediated by an enzyme, since only L-lactyl-L-amino acids were found in cheese [20]. In our work, the synthetic product Lac-Glu (**8**), synthesized from L-lactic acid and L-glutamic acid ester [19], contained a small amount of a minor diastereoisomer, likely D-Lac-L-Glu. We verified that this diastereoisomer was not present in soy sauce.

Suc-Glu (**6**) was already known as an intermediate in the arginine catabolic pathway of bacteria such as *Pseudomonas aeruginosa* [25]. A succinyl transferase enzyme (EC 2.3.1.109) is involved and is considered to be specific to arginine (Arg) and ornithine, according to the KEGG database [26][27]. However, it leaves the possibility open that other succinyl amino acids may occur in fermented products. Thus, a commercial mixture of the 20 amino acids was reacted with succinic anhydride and analyzed by UPLC/MS. In the negative-ion ESI mode, all succinyl amino acids showed a loss of 100 Da (loss of the succinyl residue). Taking advantage of this fragmentation, we rapidly developed an SRM method to analyze diluted soy sauce without any sample preparation. In addition to the two major compounds Suc-Arg and Suc-Glu (**6**), we detected significant amounts of succinylated histidine, serine, valine, leucine, isoleucine, phenylalanine, and tryptophane (data available as *Supplementary Material*). Even if the identification of many succinyl amino acids remains tentative, we can postulate that Suc-Arg and Suc-Glu (**6**) may arise from the arginine catabolic pathway, but that the succinyl transferase present in *Aspergillus oryzae* may also act on other free amino acids.

Conclusions. – During this work, we showed the natural occurrence of many derivatives of glutamic acid, many of which are claimed to be taste-active. In particular, Suc-Glu (**6**) and Lactyl-Glu (**8**) were shown to occur in a food product for the first time.

The authors thank *Robert Brauchli* for the NMR signals assignment, *Daniel Grenno* for LC/ToF recordings, and Dr. *Elise Sarrazin* for preliminary experiments. This article is dedicated to Dr. *Sina Escher*, who first identified many compounds described here in Parmesan cheese, and to *François Benzi*, flavorist, for his continuous support.

Experimental Part

General. '*Less Salt*' soy sauce (salt 8.4%, *Kikkoman Corp.*, D-Düsseldorf) was purchased from a local grocery store. High-performance liquid chromatography (HPLC)-grade MeCN and *Milli-Q* H_2O were used, and all other reagents were of anal. grade. UPLC/MS-Grade solvents were purchased from *BioSolve* (NE-Valkenswaard).

Solid-Phase Extraction (SPE) *Preparation of Soy Sauce.* An *Oasis MAX* cartridge (6 ml/500 mg, 60 μm, *Waters*, Milford, MA, USA) was conditioned and equilibrated with MeOH (10 ml) and then deionized (DI) H_2O (10 ml). Soy sauce (0.3 ml) was diluted in DI H_2O (50 ml), and then loaded onto the cartridge with a flow of 5–8 ml/min. Then 5% aq. NH_3 soln. (20 ml) was percolated at 1.5–2.5 ml/min to bind the acidic components to the resin. The cartridge was then rinsed with DI H_2O (80 ml) and dried by suction. It was rinsed with CH_2Cl_2/MeOH 95:5 (*v*/*v*; 6 ml) and carefully dried. The acidic components were eluted with 5% HCOOH (6 ml) in H_2O. The eluent was injected into the UPLC/MS.

Instruments. A system consisting of an *Acquity UPLC*® (*Waters*) and a *LXQ* mass spectrometer (*Thermo*, San Jose, CA, USA) was used for the full-scan and product ion scan experiments. A system consisting of an *Acquity UPLC*® and a *TSQ Quantum Ultra AM* mass spectrometer (*Thermo*) was used for SRM experiments.

UPLC/MS Method (Qual., *LXQ*). The column was a *Waters Acquity UPLC BEH C18* (1.7 μm 100 × 2.1 mm I. D., 25°). Solvent *A* was 0.1% HCOOH in H_2O, and solvent *B* was 0.1% HCOOH in MeCN, eluted at 0.3 ml/min in gradient mode: 0% *B* isocratic, 0–0.5 min; 0–10% *B*, 0.5–4.0 min; 10–100% *B*, 4.00–9.00 min; 100% *B* isocratic, 9.0–10.0 min; equilibration for 2 min at 0% *B*. The injection volume was 3 μl. MS: source-heated electrospray ionization (H-ESI) mass spectrometer operated in the positive- and the negative-ion mode. In the positive mode: source voltage, 4000 V, vaporizer temp., not heated, sheath gas flow rate, 50 arb. units; aux. gas flow rate, 5 arb. units; sweep gas flow rate, 0 arb. units; heated cap. temp., 325°; source CID, 5 V. In the negative-ion mode: source voltage, 5000 V; vaporizer temp., not heated; sheath gas flow rate, 50 arb. units; aux. gas flow rate, 5 arb. units; sweep gas flow rate, 0 arb. units; heated cap. temp., 325°; source CID, 5 V. Scan event *1*, full scan [130–800]; scan event *2*; data-dependent MS/MS of the 1st most intense ion from scan event *1*; collision energy, 35 V.

UPLC/MS Method (Quant., *TSQ*). The column was a *Waters Acquity UPLC HSS T3* (1.8 μm 100 × 2.1 mm i.d., 25°). Solvent *A* was 0.1% HCOOH in *Milli-Q* H_2O. The eluting conditions were the same as by the qual. method, except for the solvent gradient: 0% *B* isocratic, 0–0.5 min; 0–10% *B*, 0.5–4.0 min; 10–25% *B*, 4.00–6.00 min; 25–100% *B*, 6.00–9.00 min; 100% *B* isocratic, 9.0–10.0 min, equilibration for 2 min at 0% *B*. Injection volume was 3 μl. The acidic fraction as obtained from SPE was injected. A dilution factor of 20 was applied for the calculation regarding soy sauce.

MS: Source H-ESI operated in the negative-ion mode, spray voltage, 3900 V; vaporizer temp. 300°; sheath gas pressure, 40 arb. units; aux. gas pressure, 20 arb. units; ion sweep gas pressure, 2.0 arb. units; heated cap. temp., 325°; source CID, 5 V; Q2 Ar gas pressure, 0.8 mTorr. Data acquisition: Q1 and Q3 peak width, 0.7 (nominal mass resolution); 0.5-Da scan width; scan time, 0.05 s; and 25-V collision energy. Transitions used for quantitation in the negative mode: 275 → 128/146 (Glu-Glu, γGlu-Glu), 243 → 109/127 (pGlu-Asp), 188 → 102/128 (Ac-Glu), 257 → 128/82 (pGlu-Glu), 246 → 98/128 (Suc-Glu), 218 → 88/128 (Lac-Glu), 225 → 82/112 (pGlu-Pro), 259 → 130/128 (γGlu-Ile, γGlu-Leu), 293 → 164/128 (γGlu-Phe), 241 → 82/141 (pGlu-Ile, pGlu-Leu), 275 → 127/109 (pGlu-Phe), 202 → 143/43 (Lac-Leu).

Source H-ESI operated in positive-ion mode; spray voltage, 3700 V; vaporizer temp., 120°; sheath gas pressure, 30 arb. units; aux. gas pressure, 20 arb. units; ion sweep gas pressure 2.0 arb. units; heated cap. temp., 350°; source CID, 5 V; Q2 Ar gas pressure, 0.8 mTorr. Data acquisition: Q1 and Q3 peak width, 0.7 (nominal mass resolution); 0.5-Da scan width, scan time, 0.05 s, and 30-V collision energy. Transitions used for quantitation in the positive mode: 277 → 84/130 (Glu-Glu, γGlu-Glu), 245 → 84/74 (pGlu-Asp), 190 → 84/130 (Ac-Glu), 259 → 84/130 (pGlu-Glu), 248 → 138/84 (Suc-Glu), 220 → 84/130 (Lac-Glu), 227 → 84/70 (pGlu-Pro), 261 → 86/84 (γGlu-Ile, γGlu-Leu), 295 → 120/84 (γGlu-Phe), 243 → 86/84 (pGlu-Ile, pGlu-Leu), 277 → 120/84 (pGlu-Phe), 204 → 86/84 (Lac-Leu).

The calibration curves were obtained from solns. of 0.1–100 ppm of the synthetic reference products (prepared as described below) and prepared by consecutive dilutions in DI H_2O. Quadratic fitting and 1/× weighting gave $r^2 > 0.995$ using *XCalibur* software (*Thermo*).

Evidence for the New Natural Occurrence of αGlu-Glu (**1**)*, Ac-Glu* (**5**)*, Suc-Glu* (**6**)*, and Lac-Glu* (**8**) (*TSQ*). The ratio between the transitions for compounds **1**, **5**, and **6** was determined on the *TSQ* instrument from the calibration injections at 0.1–100 ppm (8 points) using *XCalibur* software. For compound **1** in the negative-ion mode, with the transition 275 → 128 being normalized to 100 in intensity, the transition 275 → 146 represented 59 ± 3 for the eight injections. In soy sauce extract, it was at 63. In the positive-ion mode, 277 → 84 (100), 130(43 ± 4); in soy sauce 277 → 84 (100), 130 (42). αGlu-Glu (**1**) was clearly present in soy sauce according to the guidelines of the *IOFI* [14]. Compound **5**: negative-ion mode 188 → 102 (100),128 (46 ± 3); in soy sauce 188 → 102 (100), 128 (48); positive-ion mode: 190 → 84 (100), 130 (24 ± 2); in soy sauce 190 → 84 (100), 130 (25). Ac-Glu (**5**) was clearly present in soy sauce according to *IOFI* guidelines. Compound **6**: negative-ion mode 246 → 98 (100), 128 (84 ± 7); in soy sauce 246 → 98 (100), 128 (92); positive-ion mode: 248 → 138 (100), 84 (95 ± 2); in soy sauce 248 → 138 (100), 84 (84). Suc-Glu (**6**) was clearly present in soy sauce according to *IOFI* guidelines. Compound **8**: negative-ion mode 218 → 128 (100), 88 (30 ± 5); in soy sauce 218 → 128 (100), 128 (30); positive-ion mode: 220 → 84 (100), 130 (12 ± 2); in soy sauce 248 → 84 (100), 130 (13). Lac-Glu (**8**) was clearly present in soy sauce according to *IOFI* guidelines.

TOF-MS Recordings. A *1200 Series HPLC* system and an *Agilent G1969A* ToF MS were used with multimode source. The column was the same *HSS T3* as described above (1.8 μm 100 × 2.1 mm i.d., 60°).

Solvents *A* and *B* were the same as described above. Flow rate was 0.5 ml/min in gradient mode: 0% *B* isocratic, 0–1.0 min; 0–100% *B*, 1.0–10.0 min; equilibration, for 2 min at 0% *B*; 0.5 ml/min gradient, 100% *A*, 1 min, to 100% *B* in 8 min; *B* 100% at 10 min; to 0% at 12 min post equilibration, 1 min. Injection of 1 µl. MS: Source-operated in ESI mode. High resolution: 3 ppm accuracy. Fragmentor, 140. Scan range, 103–1100; online standard for mass adjustment.

Reference Compounds. All amino acid derivatives were of L-configuration. L-Lactic acid was purchased from *Fluka* (CH-Buchs).

Products αGlu-Glu (**1**), γGlu-Glu (**2**), pGlu-Val (**11**), γGlu-Leu (**13**), γGlu-Phe (**14**), and pGlu-Phe (**15**) were purchased from *Bachem* (CH-Bubendorf); citric acid (**3**) and Ac-Glu (**5**) were purchased from *Sigma-Aldrich* (CH-Buchs). The synthesis of Suc-Glu (**6**) and its spectral data were described by *Frerot* and *Benzi* [12]. The synthesis of L-Lac-Glu (**8**) and L-Lac-Leu (**20**), and their spectral data were described by *Frerot* and *Escher* [19].

The syntheses of pyroglutamyl dipeptides pGlu-Asp (**4**), pGlu-Glu (**7**), pGlu-Pro (**8**), pGlu-Ile (**9**), and pGlu-Leu (**10**) were carried out in two steps, as exemplified by the preparation of **7**.

pGlu-Glu (=*5-Oxoprolyl-*L*-glutamic Acid*; **7**). *Step 1* (coupling): *N*-[(benzyloxy)carbonyl]-pyroglutamic acid (*Z*-pGlu-OH; 2.63 g, 10 mmol; *Novabiochem*, Läufelfingen), glutamic acid dibenzyl ester *p*-toluenesulfonate salt (H-Glu(OBzl)-OBzl; 1.1 equiv.; *Novabiochem*), and *PyBOP*® (1 equiv.; *Novabiochem*) were diluted in CH_2Cl_2 (100 ml) before EtN^iPr_2 (*Sigma Aldrich*) was added. The mixture was stirred overnight. AcOEt (250 ml) was added, and the mixture was washed with 5% $KHSO_4$, 5% $NaHCO_3$, and brine, and dried and evaporated to give a pale-yellow viscous oil. Flash chromatography over silica gel (SiO_2; *Puriflash*, 300 g; *Interchim*, F-Montluçon) was performed with cyclohexane/AcOEt and gave pure Z-pGlu-Glu(OBzl)-OBzl as a white solid (3.63 g, 63%). *Step 2* (hydrogenolysis): Z-pGlu-Glu(OBzl)-OBzl was dissolved in CH_2Cl_2/EtOH/H_2O 10 : 12 : 4 (26 ml/mmol), and 5% Pd/C (1 g, *Fluka*) was added. The mixture was shaken for 2–6 d under H_2. H_2O (*ca.* 100 ml) was added, and the mixture was washed 3 × with CH_2Cl_2. The hydroalcoholic soln. was concentrated *in vacuo*, H_2O (200 ml) was added, and the soln. was freeze-dried to yield a white powder (1.62 g, 98%). ^{1}H-NMR (400 MHz, D_2O): 1.90–2.00 (*m*, 1 H); 2.07–2.19 (*m*, 2 H); 2.37 (*t*, *J* = 7.6, 2 H); 2.41–2.48 (*m*, 2 H); 2.50–2.60 (*m*, 1 H); 4.21–4.25 (*m*, 1 H); 4.34–4.39 (*m*, 1 H). ^{13}C-NMR (125 MHz, D_2O): 28.0 (*t*); 29.1 (*t*); 32.2 (*t*); 33.5 (*t*); 56.2 (*d*); 59.9 (*d*); 177.7 (*s*); 179.3 (*s*); 180.5 (*s*); 185.4 (*s*).

pGlu-Asp (=*5-Oxoprolyl-*L*-aspartic Acid*; **4**). Overall yield: 82%. ^{1}H-NMR (400 MHz, D_2O): 2.06–2.15 (*m*, 1 H); 2.35–2.48 (*m*, 2 H); 2.50–2.60 (*m*, 1 H); 2.79 (*dd*, *J* = 16.5, 7.7, 1 H); 2.90 (*dd*, *J* = 16.5, 5.0, 1 H); 4.35 (*dd*, *J* = 9.1, 5.0, 1 H); 4.59 (*dd*, *J* = 7.7, 5.0, 1 H). ^{13}C-NMR (100 MHz, D_2O): 28.0 (*t*); 32.2 (*t*); 40.0 (*t*); 54.0 (*d*); 60.0 (*d*); 177.2 (*s*); 178.8 (*s*); 179.1 (*s*); 185.3 (*s*).

pGlu-Pro (=*5-Oxoprolylproline*; **9**). Overall yield: 91%. ^{1}H-NMR (360 MHz, D_2O): 2.00–2.09 (*m*, 4 H); 2.28–2.36 (*m*, 1 H); 2.44 (~*t*, *J* = 7.9, 2 H); 2.57–2.68 (*m*, 1 H); 3.58–3.67 (*m*, 1 H); 3.68–3.75 (*m*, 1 H); 4.46 (*dd*, *J* = 7.9, 4.8, 1 H); 4.70 (*dd*, *J* = 9.1, 5.0, 1 H). ^{13}C-NMR (90 MHz, D_2O): 26.8 (*t*); 27.5 (*t*); 31.6 (*t*); 32.1 (*t*); 49.9 (*t*); 58.3 (*d*); 62.5 (*d*); 175.8 (*s*); 178.8 (*s*); 185.0 (*s*).

pGlu-Ile (=*5-Oxoprolylisoleucine*; **16**). Overall yield: 90%. ^{1}H-NMR (360 MHz, D_2O): 0.88 (*t*, *J* = 7.4, 3 H); 0.93 (*d*, *J* = 6.9, 3 H); 1.13–1.26 (*m*, 1 H); 1.39–1.51 (*m*, 1 H); 1.85–1.96 (*m*, 1 H); 2.04–2.14 (*m*, 1 H); 2.40–2.46 (*m*, 2 H); 2.48–2.60 (*m*, 1 H); 4.22 (*d*, *J* = 6.1, 1 H); 4.40 (*dd*, *J* = 8.9, 5.1, 1 H). ^{13}C-NMR (90 MHz, D_2O): 13.6 (*q*); 18.0 (*q*); 27.6 (*t*); 28.1 (*t*); 32.2 (*t*); 39.4 (*d*); 59.8 (*d*); 62.0 (*d*); 177.5 (*s*); 179.7 (*s*); 185.3 (*s*).

pGlu-Leu (=*5-Oxoprolylleucine*; **17**). Overall yield: 86%. ^{1}H-NMR (360 MHz, D_2O): 0.91 (br. *d*, *J* = 6.0, 3 H); 0.93 (br. *d*, *J* = 6.0, 3 H); 1.58–1.65 (*m*, 3 H); 2.06–2.16 (*m*, 1 H); 2.41–2.47 (*m*, 2 H); 2.50–2.62 (*m*, 1 H); 4.21–4.28 (*m*, 1 H); 4.36 (*dd*, *J* = 9.1, 5.0, 1 H). ^{13}C-NMR (90 MHz, D_2O): 23.6 (*q*); 25.3 (*q*); 27.5 (*d*); 28.0 (*t*); 32.2 (*t*); 43.3 (*t*); 56.8 (*d*); 59.9 (*d*); 177.1 (*s*); 182.6 (*s*); 185.2 (*s*).

Lac-Val (**15**) was synthesized in two steps according to *Frerot* and *Escher* [19] from L-lactic acid and H-Val-OBzl tosylate salt (*Bachem*), with an overall yield of 56% after hydrogenolysis. ^{1}H-NMR (400 MHz, D_2O): 0.91 (*d*, *J* = 6.9, 3 H); 0.94 (*d*, *J* = 6.9, 3 H); 1.39 (*d*, *J* = 6.9, 3 H); 2.17 (*m*, 1 H); 4.15 (*d*, *J* = 5.7, 1 H); 4.30 (*q*, *J* = 6.9, 1 H). ^{13}C-NMR (100 MHz, D_2O): 20.0 (*q*); 21.6 (*q*); 22.7 (*q*); 33.4 (*d*); 62.2 (*d*); 70.8 (*d*); 180.0 (*s*); 180.3 (*s*).

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