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Stereoselective Synthesis of Amides Sharing the Guanosine 5′-Monophosphate Scaffold and Umami Enhancement Studies Using Human Sensory and hT1R1/rT1R3 Receptor Assays

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ABSTRACT: Recent studies led to the identification of umami-enhancing (*S*)-N^2-(1-carboxyethyl)- and (*S*)-N^2-(1-alkylamino)-carbonylalkyl)guanosine 5′-monophosphates that, together with their sensorially inactive (*R*)-stereoisomers, were found to be formed upon Maillard-type glycation of guanosine 5′-monophosphate (5′-GMP) with 1,3-dihydroxyacetone or glyceraldehyde, respectively. As the efficiency of this Maillard-type procedure to generate the amidated derivatives is limited by the low solubility and reactivity of long-chain alkyl amines as well as by the tedious separation of the diastereomers formed, a versatile synthesis for the (*R*)- and (*S*)-configured amides of N^2-carboxyalkylated guanosine 5′-monophosphate was developed. Sensory evaluation of a series of N^2-(1-alkylamino)carbonylalkyl)guanosine 5′-monophosphates revealed β-values for umami enhancement between 0.1 and 7.7 and identified a strong influence of the stereochemistry as well as the chain length of the N^2-substituent on the umami-enhancing activity. The observed sensory impact of the (*S*)-configured isomers was confirmed by recording the enhancing effect of these nucleotides on the L-glutamate-induced response of the functionally expressed T1R1/T1R3 umami receptor in a cell-based assay, thus underscoring the stereospecifity of the umami taste receptor binding site.

KEYWORDS: guanosine 5′-monophosphate, taste enhancer, umami, T1R1, T1R3, ribonucleotides, Maillard reaction

INTRODUCTION

The perception of smell and taste is mediated by the activation of specific chemoreceptor cells by volatile and nonvolatile molecules entering the nose and the oral cavity, respectively. Sweetness, bitterness, sourness, and saltiness are long accepted as basic taste qualities. Although L-glutamate was identified in seaweed broth as the key stimulus of umami taste as early as 1908, the classification of umami as the fifth basic taste has been only recently promoted by cloning of a special amino acid taste receptor in 2002 and the discovery of cell populations in the oral cavity especially dedicated to stimuli of only one taste quality.[1−5] By means of expression studies, the proteins T1R1 and T1R3 were shown to assemble into a functional heteromeric receptor for L-amino acids, if the subunits were from mice, or specifically for L-glutamate, if they were of human origin.[1,2] This selective L-glutamate response is a well-known hallmark of umami taste.[5,6] The T1R1 subunit of the heteromer was shown to interact with the L-glutamate molecule as a heterologous functional expression of chimeric receptors consisting of human T1R1, and rodent T1R3 was highly selective for L-glutamate and consistent with psychophysical evidence.[1] The proposed binding site for L-glutamate is located in a special binding domain, that is, the Venus flytrap domain of the amino-terminal extracellular domain of T1R1. Mechanistically, it is proposed that L-glutamate binding to the hinge region of the Venus flytrap domain causes a closure of the domain.[2,5,7]

Moreover, the heteromeric T1R1/T1R3 receptor was found to show another signature characteristic for umami taste, that is, the ability to give synergistically enhanced responses in the presence of purine 5′-ribonucleotides including inosine 5′-monophosphate, **1** (5′-IMP, Figure 1) and guanosine 5′-monophosphate (5′-GMP, **2**), respectively.[5,7] Interestingly, inosine 5′-monophosphate (**1**) was demonstrated to interact at a different position of the Venus fly trap domain and is supposed to stabilize the closed, active conformation induced upon L-glutamate binding.[7] In consequence, the T1R1−T1R3 heteromer is today referred to as the umami receptor.[5]

Aimed at identifying the structural requirements for taste enhancement of 5′-ribonucleotides, a series of synthetic experiments were undertaken to obtain structural analogues with superior taste enhancement properties when compared to the parent nucleotides.[8−10] First approaches showed an enhanced umami activity of thioalkylated nucleotides such as 2-methylthio 5′-IMP, **3** (Figure 1), exhibiting an 8-fold increased umami impact when compared to the parent nucleotide, whereas the activity of N^2-alkylated nucleotides such as N^2-methyl 5′-GMP (**4**) and N^2-dimethyl 5′-GMP (**5**) were not significantly different from those of 5′-GMP.[8−10] On the basis of these findings, a comprehensive synthetic study investigated the umami-enhancing activity of a series of 2-substituted inosine 5′-monophosphates and revealed 2-alkylmercapto 5′-IMP derivatives as the most potent class of molecules; for example, 2-furfurylthio 5′-IMP, **6** (Figure 1), was reported to reach a maximum of 17 times the activity of **1**.[8,11]

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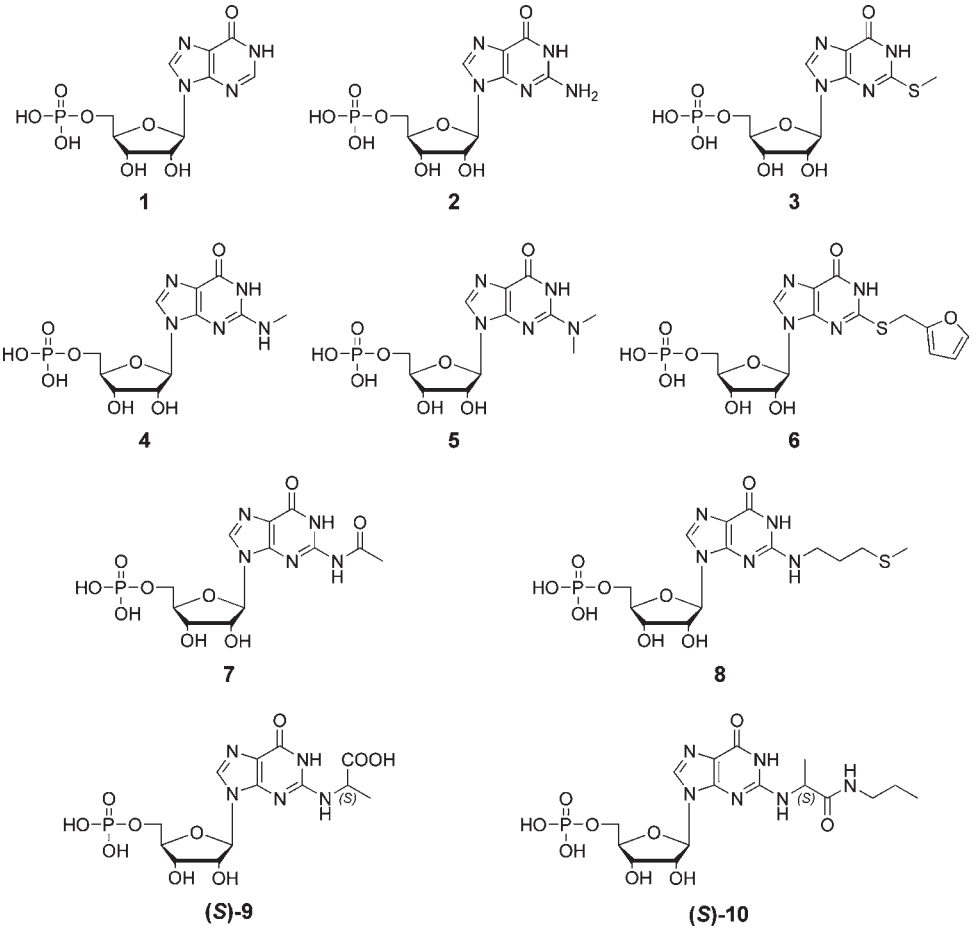


Figure 1. Chemical structures of umami-enhancing purine ribonucleotides: inosine 5′-monophosphate (5′-IMP, **1**), guanosine 5′-monophosphate (5′-GMP, **2**), 2-methylthio 5′-IMP (**3**), N^2-methyl 5′-GMP (**4**), N^2-dimethyl 5′-GMP (**5**), 2-furfurylthio 5′-IMP (**6**), N^2-acetyl 5′-GMP (**7**), N^2-(3-methylthiopropyl) 5′-GMP (**8**), (*S*)-N^2-(1-carboxyethyl) 5′-GMP ((*S*)-**9**), and (*S*)-N^2-((1-(*N*-propylamino)carbonyl)ethyl) 5′-GMP ((*S*)-**10**).

In addition, N^2-acylated 5′-GMP derivatives such as N^2-acetylguanosine 5′-monophosphate, **7** (Figure 1), were reported to exhibit an intensified umami taste profile when compared to **2**.[12,13] Moreover, a series of N^2-alkylated and N^2-acylated 5′-GMP derivatives was synthesized and sensorially evaluated, thus demonstrating that the umami-enhancing activity of the molecules was strongly dependent on the carbon chain length and the presence/position of a sulfur heteroatom as well as the presence of an α-CO group in the N^2-substituent; for example, N^2-(3-methylthiopropyl) 5′-GMP, **8** (Figure 1), reached a maximum activity of 5.7 times that found for **1**.[14]

Very recently, sensory-directed fractionation of commercial yeast extracts revealed (*S*)-N^2-(1-carboxyethyl)guanosine 5′-monophosphate, (*S*)-**9** (Figure 1), as a previously not reported umami-enhancing nucleotide formed upon Maillard reaction of 5′-GMP and glyceraldehyde.[15,16] Systematic model reactions performed with 5′-GMP and a homologous series of C_3−C_6-monosaccharides led to the discovery of a series of (*R*)/(*S*)-N^2-(1-carboxyalkyl)guanosine 5′-monophosphates.[15,16] When these model reactions were performed in the presence of an amino compound, (*R*)/(*S*)-N^2-(1-alkylamino)carbonylalkyl)-guanosine 5′-monophosphates were obtained; for example, (*S*)-N^2-((1-(*N*-propylamino)carbonyl)ethyl) 5′-GMP, (*S*)-**10** (Figure 1), was obtained from the Maillard reaction of 5′-GMP, dihydroxyacetone, and *n*-propylamine.[16] For all of these 5′-GMP derivatives exhibiting an α-aminocarboxylic acid or an α-aminocarboxylic acid amide moiety, respectively, the (*S*)-configured isomers showed higher umami-enhancing impact, whereas the corresponding (*R*)-isomers were almost inactive.

The objective of the present study was to synthetically prepare a broad range of N^2-(1-alkylamino)carbonylalkyl) 5′-GMP derivatives and, after purification, to functionally characterize their umami-enhancing activity by means of human sensory analysis as well as cell-based taste receptor assay.

MATERIALS AND METHODS

Chemicals and Materials. Unless stated otherwise, all chemicals were obtained from Sigma-Aldrich (Steinheim, Germany) and were of puriss grade; formic acid, disodium hydrogen phosphate dihydrate, potassium dihydrogen phosphate, and RP-18 bulk material (LiChroprep RP-18, 25−40 μm) were from Merck KGaA (Darmstadt, Germany). Deuterated solvents and sodium deuteroxide (40% w/w solution in D_2O) were obtained from Euriso-Top (Gif-Sur-Yvette, France); HPLC grade solvents were from Mallinckrodt Baker (Griesheim, Germany), and membrane filter disks (0.45 μm) were purchased from Satorius AG (Goettingen, Germany). Water used for chromatography was purified by means of a Milli-Q Advantage A10 water purification system (Millipore, Molsheim, France), and bottled water (Evian) was used for sensory analysis.

Sensory Analyses. *Sensory Training and Precautions Taken for Sensory Analysis.* Thirteen subjects (11 women and 2 men, aged 22−30 years), who gave consent to participate in the sensory tests of the present investigation and have no history of known taste disorders, were trained to evaluate the umami taste quality of aqueous solutions of monosodium L-glutamate (3.0 mmol/L), a binary mixture of monosodium L-glutamate (3.0 mmol/L) and guanosine 5′-monophosphate (0.1 mmol/L), and an aqueous solution of disodium succinate (5 mmol/L) as reported recently.[15] The assessors had participated earlier at regular intervals for at least 12 months in sensory experiments and were, therefore, familiar with the techniques applied. To prevent cross-modal interactions with olfactory inputs, the panelists wore nose clips. Prior to sensory analysis, the test compounds were confirmed by GC-MS and ion chromatographic analyses to be essentially free of the solvents and buffer compounds used.[15] To minimize the uptake of any potentially harmful compound, all of the sensory analyses were performed by using the sip-and-spit method, which means the test materials were not swallowed but expectorated.

Determination of Taste-Enhancing Activity (β Values). The activity of the nucleotide derivatives to synergistically enhance the umami taste of MSG was determined by means of a paired-choice comparison test performed in triplicates.[17] To achieve this, a binary solution (pH 6.0) containing the test nucleotide (0.05 mmol/L) and MSG (3 mmol/L) in water ("fixed sample") was compared to a series of aqueous solutions containing constant levels of MSG (3 mmol/L) and logarithmically (30% intervals) increasing concentrations of inosine 5′-monophosphate ("reference samples"). In each sensory session the assessors were asked to evaluate five sample pairs, presented in randomly coded cups, and to identify the sample exhibiting the stronger umami taste using a forced-choice methodology. The data obtained were converted into the percentage of positive responses, whereas judgments were considered to be positive if the fixed sample had the stronger umami taste. By applying the probit method, the point of equivalent umami taste intensity (50% value) was determined and expressed in concentration of 5′-IMP (**1**). The so-called β value of each test nucleotide, related to the reference 5′-IMP (**1**), was calculated according to the following equation: $v = \beta v'$, wherein v represents the 5′-IMP concentration at the point of umami taste equality (mmol/L) and v' the concentration of the test nucleotide (mmol/L).

Preparation of N^2-(1-Carboxyethyl)guanosine 5′-Monophosphate (9). Following a literature protocol with some modifications,[15] a mixture of guanosine 5′-monophosphate (2 mmol) and 1,3-dihydroxyacetone dimer (3 mmol) in phosphate buffer (1 mol/L, pH 7.0; 5 mL) was heated for 24 h at 70 °C; the crude mixture was diluted with water (25 mL) and, then, purified by means of RP-MPLC. The effluent of each peak showing absorption at $\lambda = 260$ nm was collected separately and, after removal of the solvent in vacuum, was lyophilized twice. The diastereomeric target compounds (*R*)-**9** and (*S*)-**9** showed identical LC-MS and 1D/2D NMR data as reported recently and were confirmed by cochromatography with the authentic reference substances.[15]

Synthesis of Amides 10−19 from N^2-(1-Carboxyethyl)-guanosine 5′-Monophosphate (9). A solution of (*R*)- or (*S*)-**9** (0.1 mmol), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (0.5 mmol), and the corresponding amine (2.0 mmol) or its hydrochloric acid salt (2.0 mmol) in water (4 mL) was adjusted to pH 5.0 using hydrochloric acid (1 mol/L) or sodium hydroxide (1 mol/L), respectively. The solution was stirred for 4 h at room temperature; the pH value was maintained at 5.0 by the addition of hydrochloric acid (0.1 mol/L) or sodium hydroxide (0.1 mol/L), respectively. After complete conversion of (*R*)- or (*S*)-**9** monitored by means of analytical RP-HPLC, the target compounds were isolated by preparative RP-HPLC, followed by rechromatography. The isolates were separated from solvent under vacuum and, after dilution with water, freeze-dried twice to obtain the corresponding amides **10**−**19** of (*R*)/(*S*)-N^2-(1-carboxyethyl)guanosine 5′-monophosphate as amorphous white powders with a purity of >98% (HPLC, ^{1}H NMR).

*(R)-N²-((1-(N-Propylamino)carbonyl)ethyl)guanosine 5′-monophosphate, (R)-**10**:* TOF-MS and ^{1}H and ^{13}C NMR data were identical to those published recently.[16]

*(S)-N²-((1-(N-Propylamino)carbonyl)ethyl)guanosine 5′-monophosphate, (S)-**10**:* TOF-MS and ^{1}H and ^{13}C NMR data were identical to those published recently.[16]

*(R)-N²-((1-(N-Methylamino)carbonyl)ethyl)guanosine 5′-monophosphate, (R)-**11**:* TOF-MS, *m/z* 447.1037 (found), *m/z* 447.1035 (calcd for $[C_{14}H_{20}N_6O_9P]^-$); ^{1}H NMR (400 MHz, CD_3OD/NaOD, COSY), δ 1.39 [d, 3H, J = 7.1 Hz, H−C(3″)], 2.74 [s, 3H, H−C(1‴)], 3.97−4.04 [m, 1H, H−C(5′$_a$)], 4.06−4.13 [m, 1H, H−C(5′$_b$)], 4.15 [m, 1H, H−C(4′)], 4.40−4.48 [m, 2H, H−C(3′), H−C(2″)], 4.68 [pt, 1H, J = 4.9 Hz, H−C(2′)], 5.83 [d, 1H, J = 4.6 Hz, H−C(1′)], 8.00 [s, 1H, H−C(8)]; ^{13}C NMR (100 MHz, CD_3OD/NaOD, HMQC, HMBC, DEPT), δ 19.0 [C(3″)], 26.4 [C(1‴)], 52.7 [C(2″)], 65.1 [d, $^2J_{C,P}$ = 4.6 Hz, C(5′)], 72.3 [C(3′)], 76.0 [C(2′)], 85.6 [d, $^3J_{C,P}$ = 8.3 Hz, C(4′)], 90.2 [C(1′)], 119.8 [C(5)], 137.4 [C(8)], 153.0 [C(4)], 161.1 [C(2)], 169.7 [C(6)], 178.7 [C(1″)].

*(S)-N²-((1-(N-Methylamino)carbonyl)ethyl)guanosine 5′-monophosphate, (S)-**11**:* TOF-MS, *m/z* 447.1038 (found), *m/z* 447.1035 (calcd for $[C_{14}H_{20}N_6O_9P]^-$); ^{1}H NMR (400 MHz, CD_3OD/NaOD, COSY), δ 1.40 [d, 3H, J = 7.1 Hz, H−C(3″)], 2.74 [s, 3H, H−C(1‴)], 3.94−4.00 [m, 1H, H−C(5′$_a$)], 4.07−4.12 [m, 1H, H−C(5′$_b$)], 4.16 [m, 1H, H−C(4′)], 4.31 [pt, 1H, J = 5.2 Hz, H−C(3′)], 4.51 [q, 1H, J = 7.0 Hz, H−C(2″)], 4.60 [pt, 1H, J = 5.0 Hz, H−C(2′)], 5.83 [d, 1H, J = 4.3 Hz, H−C(1′)], 7.99 [s, 1H, H−C(8)]; ^{13}C NMR (100 MHz, CD_3OD/NaOD, HMQC, HMBC, DEPT), δ 19.1 [C(3″)], 26.4 [C(1‴)], 52.2 [C(2″)], 65.7 [d, $^2J_{C,P}$ = 4.6 Hz, C(5′)], 73.0 [C(3′)], 77.0 [C(2′)], 85.5 [d, $^3J_{C,P}$ = 8.3 Hz, C(4′)], 90.7 [C(1′)], 119.7 [C(5)], 136.9 [C(8)], 153.1 [C(4)], 161.2 [C(2)], 169.8 [C(6)], 178.3 [C(1″)].

*(R)-N²-((1-(N-Ethylamino)carbonyl)ethyl)guanosine 5′-monophosphate, (R)-**12**:* TOF-MS, *m/z* 461.1186 (found), *m/z* 461.1191 (calcd for $[C_{15}H_{22}N_6O_9P]^-$); ^{1}H NMR (400 MHz, CD_3OD/NaOD, COSY), δ 1.08 [t, 3H, J = 7.3 Hz, H−C(2‴)], 1.39 [d, 3H, J = 7.1 Hz, H−C(3″)], 3.20 [q, 2H, J = 7.2 Hz, H−C(1‴)], 3.96−4.05 [m, 1H, H−C(5′$_a$)], 4.05−4.13 [m, 1H, H−C(5′$_b$)], 4.16 [m, 1H, H−C(4′)], 4.38−4.48 [m, 2H, H−C(3′), H−C(2″)], 4.61 [pt, 1H, J = 4.8 Hz, H−C(2′)], 5.87 [d, 1H, J = 4.6 Hz, H−C(1′)], 8.06 [s, 1H, H−C(8)]; ^{13}C NMR (100 MHz, CD_3OD/NaOD, HSQC, HMBC), δ 14.9 [C(2‴)], 19.0 [C(3″)], 35.2 [C(1‴], 52.8 [C(2″)], 65.0 [d, $^2J_{C,P}$ = 4.4 Hz, C(5′)], 72.2 [C(3′], 76.4 [C(2′)], 85.6 [d, $^3J_{C,P}$ = 8.3 Hz, C(4′)], 89.9 [C(1′)], 119.7 [C(5)], 137.2 [C(8)], 153.0 [C(4)], 161.1 [C(2)], 169.7 [C(6)], 177.7 [C(1″)].

*(S)-N²-((1-(N-Ethylamino)carbonyl)ethyl)guanosine 5′-monophosphate, **12**:* TOF-MS, *m/z* 461.1190 (found), *m/z* 461.1191 (calcd for $[C_{15}H_{22}N_6O_9P]^-$); ^{1}H NMR (400 MHz, CD_3OD/NaOD, COSY), δ 1.08 [t, 3H, J = 7.3 Hz, H−C(2‴)], 1.38 [d, 3H, J = 7.1 Hz, H−C(3″)], 3.20 [q, 2H, J = 7.2 Hz, H−C(1‴)], 3.97−4.05 [m, 1H, H−C(5′$_a$)], 4.05−4.12 [m, 1H, H−C(5′$_b$)], 4.17 [m, 1H, H−C(4′)], 4.43 [pt, 1H, J = 4.9 Hz, H−C(3′)], 4.48 [q, 1H, J = 7.1 Hz, H−C(2″)], 4.55 [pt, 1H, J = 4.8 Hz, H−C(2′)], 5.90 [d, 1H, J = 4.4 Hz, H−C(1′)], 8.12 [s, 1H, H−C(8)]; ^{13}C NMR (100 MHz, CD_3OD/NaOD, HSQC, HMBC), δ 14.9 [C(2‴)], 19.0 [C(3″)], 35.2 [C(1‴], 52.6 [C(2″)], 64.9 [d, $^2J_{C,P}$ = 4.5 Hz, C(5′)], 72.1 [C(3′], 76.7 [C(2′)], 85.7 [d, $^3J_{C,P}$ = 8.5 Hz, C(4′)], 89.5 [C(1′)], 119.5 [C(5)], 137.0 [C(8)], 153.0 [C(4)], 161.1 [C(2)], 169.7 [C(6)], 177.6 [C(1″)].

*(R)-N²-((1-(N-Isopropylamino)carbonyl)ethyl)guanosine 5′-monophosphate, (R)-**13**:* TOF-MS, *m/z* 475.1354 (found), *m/z* 475.1348 (calcd for $[C_{16}H_{24}N_6O_9P]^-$); ^{1}H NMR (400 MHz, CD_3OD/NaOD, COSY), δ 1.08 [d, 3H, J = 6.6 Hz, H−C(2‴$_a$)], 1.12 [d, 3H, J = 6.6 Hz, H−C(2‴$_b$)], 1.37 [d, 3H, J = 7.1 Hz, H−C(3″)], 3.93 [sept, 1H, J = 6.6 Hz, H−C(2‴)], 3.98−4.05 [m, 1H, H−C(5′$_a$)], 4.05−4.12 [m, 1H, H−C(5′$_b$)], 4.15 [m, 1H, H−C(4′)], 4.40 [q, 1H, J = 7.1 Hz,

H−C(2″)], 4.46 [pt, 1H, J = 4.9 Hz, H−C(3′)], 4.56 [pt, 1H, J = 4.7 Hz, H−C(2′)], 5.90 [d, 1H, J = 4.4 Hz, H−C(1′)], 8.12 [s, 1H, H−C(8)]; ^{13}C NMR (100 MHz, CD_3OD/NaOD, HMQC, HMBC, DEPT), δ 18.9 [C(3″)], 22.6 [C(2‴$_{a,b}$)], 42.4 [C(1‴)], 52.8 [C(2″)], 64.8 [d, $^2J_{C,P}$ = 4.4 Hz, C(5′)], 72.1 [C(3′], 76.7 [C(2′)], 85.6 [d, $^3J_{C,P}$ = 8.6 Hz, C(4′)], 89.5 [C(1′)], 119.5 [C(5)], 137.1 [C(8)], 153.0 [C(4)], 161.1 [C(2)], 169.7 [C(6)], 178.0 [C(1″)].

*(S)-N^2-((1-(N-Isopropylamino)carbonyl)ethyl)guanosine 5′-monophosphate, (S)-**13***: TOF-MS, m/z 475.1359 (found), m/z 475.1348 (calcd for $[C_{16}H_{24}N_6O_9P]^-$); ^{1}H NMR (400 MHz, CD_3OD/NaOD, COSY), δ 1.08 [d, 3H, J = 6.6 Hz, H−C(2‴$_a$)], 1.12 [d, 3H, J = 6.6 Hz, H−C(2‴$_b$)], 1.37 [d, 3H, J = 7.1 Hz, H−C(3″)], 3.89−3.98 [m, 1H, J = 6.6 Hz, H−C(2‴)], 3.98−4.04 [m, 1H, H−C(5′$_a$)], 4.04−4.13 [m, 1H, H−C(5′$_b$)], 4.17 [m, 1H, H−C(4′)], 4.41 [t, 1H, J = 4.9 Hz, H−C(3′)], 4.47 [q, 1H, J = 7.1 Hz, H−C(2″)], 4.54 [t, 1H, J = 4.7 Hz, H−C(2′)], 5.91 [d, 1H, J = 4.4 Hz, H−C(1′)], 8.13 [s, 1H, H−C(8)]; ^{13}C NMR (100 MHz, CD_3OD/NaOD, HMQC, HMBC, DEPT), δ 18.9 [C(3″)], 22.5 [C(2‴$_{a,b}$)], 42.2 [C(1‴)], 52.5 [C(2″)], 64.7 [d, $^2J_{C,P}$ = 4.9 Hz, C(5′)], 72.0 [C(3′], 76.7 [C(2′)], 85.5 [d, $^3J_{C,P}$ = 8.4 Hz, C(4′)], 89.3 [C(1′)], 119.3 [C(5)], 136.8 [C(8)], 152.9 [C(4)], 161.0 [C(2)], 169.6 [C(6)], 176.7 [C(1″)].

*(R)-N^2-((1-(N-Cyclopropylamino)carbonyl)ethyl)guanosine 5′-monophosphate, (R)-**14***: TOF-MS, m/z 473.1205 (found), m/z 473.1191 (calcd for $[C_{16}H_{22}N_6O_9P]^-$); ^{1}H NMR (400 MHz, CD_3OD/NaOD, COSY), δ 0.50 [m, 2H, H−C(2‴$_a$), H−C(3‴$_a$)], 0.67 [m, 2H, H−C(2‴$_b$), H−C(3‴$_b$)], 1.37 [d, 3H, J = 7.1 Hz, H−C(3″)], 2.63 [m, 1H, H−C(1‴)], 3.95−4.05 [m, 1H, H−C(5′$_a$)], 4.05−4.13 [m, 1H, H−C(5′$_b$)], 4.17 [m, 1H, H−C(4′)], 4.39 [q, 1H, J = 7.1 Hz, H−C(2″)], 4.44 [pt, 1H, J = 4.9 Hz, H−C(3′)], 4.63 [pt, 1H, J = 4.9 Hz, H−C(2′)], 5.86 [d, 1H, J = 4.5 Hz, H−C(1′)], 8.06 [s, 1H, H−C(8)]; ^{13}C NMR (100 MHz, CD_3OD/NaOD, HMQC, HMBC, DEPT), δ 6.5 [C(2‴), C(3‴)], 18.8 [C(3″)], 23.3 [C(1‴)], 52.5 [C(2″)], 65.0 [d, $^2J_{C,P}$ = 4.8 Hz, C(5′)], 72.2 [C(3′], 76.3 [C(2′)], 85.7 [d, $^3J_{C,P}$ = 8.4 Hz, C(4′)], 89.8 [C(1′)], 119.6 [C(5)], 137.2 [C(8)], 153.0 [C(4)], 161.1 [C(2)], 169.7 [C(6)], 179.7 [C(1″)].

*(S)-N^2-((1-(N-Cyclopropylamino)carbonyl)ethyl)guanosine 5′-monophosphate, (S)-**14***: TOF-MS, m/z 473.1191 (found), m/z 473.1191 (calcd for $[C_{16}H_{22}N_6O_9P]^-$); ^{1}H NMR (400 MHz, CD_3OD/NaOD, COSY), δ 0.49 [m, 2H, H−C(2‴$_a$), H−C(3‴$_a$)], 0.67 [m, 2H, H−C(2‴$_b$), H−C(3‴$_b$)], 1.37 [d, 3H, J = 7.1 Hz, H−C(3″)], 2.64 [m, 1H, H−C(1‴)], 3.97−4.05 [m, 1H, H−C(5′$_a$)], 4.05−4.13 [m, 1H, H−C(5′$_b$)], 4.17 [m, 1H, H−C(4′)], 4.41 [pt, 1H, J = 4.9 Hz, H−C(3′)], 4.46 [q, 1H, J = 7.1 Hz, H−C(2″)], 4.57 [pt, 1H, J = 4.8 Hz, H−C(2′)], 5.89 [d, 1H, J = 4.4 Hz, H−C(1′)], 8.10 [s, 1H, H−C(8)]; ^{13}C NMR (100 MHz, CD_3OD/NaOD, HSQC, HMBC), δ 6.5−6.6 [C(2‴), C(3‴)], 19.0 [C(3″)], 23.3 [C(1‴)], 52.4 [C(2″)], 65.0 [d, $^2J_{C,P}$ = 4.5 Hz, C(5′)], 72.2 [C(3′], 76.7 [C(2′)], 85.7 [d, $^3J_{C,P}$ = 8.3 Hz, C(4′)], 89.7 [C(1′)], 119.5 [C(5)], 137.0 [C(8)], 153.1 [C(4)], 161.1 [C(2)], 169.8 [C(6)], 179.5 [C(1″)].

*(R)-N^2-((1-(N-Butylamino)carbonyl)ethyl)guanosine 5′-monophosphate, (R)-**15***: TOF-MS, m/z 489.1528 (found), m/z 489.1504 (calcd for $[C_{17}H_{26}N_6O_9P]^-$); ^{1}H NMR (400 MHz, CD_3OD/NaOD, COSY), δ 0.82 [t, 3H, J = 7.4 Hz, H−C(4‴), 1.24 [m, 2H, H−C(3‴)], 1.39 [d, 3H, J = 7.1 Hz, H−C(3″)], 1.45 [m, 2H, H−C(2‴)], 3.12 [dt, 1H, J = 13.7, 7.0 Hz, H−C(1‴$_a$)], 3.22 [dt, 1H, J = 13.7, 7.0 Hz, H−C(1‴$_b$)], 3.98−4.06 [m, 1H, H−C(5′$_a$)], 4.06−4.13 [m, 1H, H−C(5′$_b$)], 4.16 [m, 1H, H−C(4′)], 4.41 [q, 1H, J = 7.1 Hz, H−C(2″)], 4.46 [pt, 1H, J = 5.0 Hz, H−C(3′)], 4.57 [pt, 1H, J = 4.7 Hz, H−C(2′)], 5.88 [d, 1H, J = 4.2 Hz, H−C(1′)], 8.09 [s, 1H, H−C(8)]; ^{13}C NMR (100 MHz, CD_3OD/NaOD, HMQC, HMBC, DEPT), δ 14.2 [C(4‴)], 19.0 [C(3″)], 21.1 [C(3‴)], 32.7 [C(2‴)], 40.1 [C(1‴)], 53.1 [C(2″)], 65.0 [d, $^2J_{C,P}$ = 4.6 Hz, C(5′)], 72.2 [C(3′], 76.7 [C(2′)], 85.6 [d, $^3J_{C,P}$ = 8.3 Hz, C(4′)], 90.0 [C(1′)], 119.8 [C(5)], 137.3 [C(8)], 153.0 [C(4)], 161.2 [C(2)], 169.8 [C(6)], 177.1 [C(1″)].

*(S)-N^2-((1-(N-Butylamino)carbonyl)ethyl)guanosine 5′-monophosphate, (S)-**15***: TOF-MS, m/z 489.1512 (found), m/z 489.1504 (calcd for $[C_{17}H_{26}N_6O_9P]^-$); ^{1}H NMR (400 MHz, CD_3OD/NaOD, COSY), δ 0.85 [t, 3H, J = 7.3 Hz, H−C(4‴), 1.26 [dq, 2H, J = 14.5, 7.3 Hz, H−C(3‴)], 1.39 [d, 3H, J = 7.1 Hz, H−C(3″)], 1.45 [m, 2H, H−C(2‴)], 3.17 [t, 2H, J = 7.0 Hz, H−C(1‴)], 3.97−4.05 [m, 1H, H−C(5′$_a$)], 4.05−4.13 [m, 1H, H−C(5′$_b$)], 4.16 [m, 1H, H−C(4′)], 4.40 [pt, 1H, J = 5.0 Hz, H−C(3′)], 4.46−4.55 [m, 2H, H−C(2′), H−C(2″)], 5.89 [d, 1H, J = 4.2 Hz, H−C(1′)], 8.11 [s, 1H, H−C(8)]; ^{13}C NMR (100 MHz, CD_3OD/NaOD, HSQC, HMBC), δ 14.1 [C(4‴)], 19.0 [C(3″)], 21.0 [C(3‴)], 32.5 [C(2‴)], 40.0 [C(1‴)], 52.7 [C(2″)], 65.0 [d, $^2J_{C,P}$ = 3.6 Hz, C(5′)], 72.1 [C(3′], 76.9 [C(2′)], 85.7 [d, $^3J_{C,P}$ = 8.3 Hz, C(4′)], 89.8 [C(1′)], 119.6 [C(5)], 137.0 [C(8)], 153.1 [C(4)], 161.1 [C(2)], 169.8 [C(6)], 177.7 [C(1″)].

*(S)-N^2-((1-(N-Isobutylamino)carbonyl)ethyl)guanosine 5′-monophosphate, (S)-**16***: TOF-MS, m/z 489.1493 (found), m/z 489.1504 (calcd for $[C_{17}H_{26}N_6O_9P]^-$); ^{1}H NMR (400 MHz, CD_3OD/NaOD, COSY), δ 0.81 [dd, 6H, J = 8.6 Hz, 6.7 Hz, H−C(3‴), H−C(4‴)], 1.40 [d, 3H, J = 7.1 Hz, H−C(3″)], 1.75 [m, 1H, H−C(2‴)], 2.96 [dd, 1H, J = 13.2 Hz, 7.0 Hz, H−C(1‴$_a$)], 3.03 [dd, 1H, J = 13.2 Hz, 6.9 Hz, H−C(1‴$_b$)], 3.98−4.05 [m, 1H, H−C(5′$_a$)], 4.05−4.11 [m, 1H, H−C(5′$_b$)], 4.17 [m, 1H, H−C(4′)], 4.41 [pt, 1H, J = 5.0 Hz, H−C-(3′)], 4.48−4.53 [m, 2H, H−C(2′), H−C(2″)], 5.90 [d, 1H, J = 4.2 Hz, H−C(1′)], 8.13 [s, 1H, H−C(8)]; ^{13}C NMR (100 MHz, CD_3OD/NaOD, HSQC, HMBC), δ 19.0 [C(3‴)], 20.4−20.5 [C(3‴), C(4‴)], 29.6 [C(2‴)], 47.7 [C(1‴)], 52.7 [C(2″)], 64.9 [d, $^2J_{C,P}$ = 4.3 Hz, C(5′)], 72.0 [C(3′], 76.8 [C(2′)], 85.6 [d, $^3J_{C,P}$ = 8.4 Hz, C(4′)], 89.6 [C(1′)], 119.5 [C(5)], 137.0 [C(8)], 153.0 [C(4)], 161.1 [C(2)], 169.8 [C(6)], 177.9 [C(1″)].

*(R)-N^2-((1-(N-Pentylamino)carbonyl)ethyl)guanosine 5′-monophosphate, (R)-**17***: TOF-MS, m/z 503.1682 (found), m/z 503.1661 (calcd for $[C_{18}H_{28}N_6O_9P]^-$); ^{1}H NMR (400 MHz, CD_3OD/NaOD, COSY), δ 0.82 [t, 3H, J = 7.4 Hz, H−C(5‴), 1.24 [m, 4H, H−C(3‴), H−C(4‴)], 1.39 [d, 3H, J = 7.1 Hz, H−C(3″)], 1.45 [m, 2H, H−C(2‴)], 3.12 [dt, 1H, J = 13.5, 7.0 Hz, H−C(1‴$_a$)], 3.22 [dt, 1H, J = 13.9, 7.0 Hz, H−C(1‴$_b$)], 3.98−4.06 [m, 1H, H−C(5′$_a$)], 4.06−4.13 [m, 1H, H−C(5′$_b$)], 4.16 [m, 1H, H−C(4′)], 4.41 [q, 1H, J = 7.1 Hz, H−C(2″)], 4.46 [pt, 1H, J = 5.0 Hz, H−C(3′)], 4.57 [pt, 1H, J = 4.7 Hz, H−C(2′)], 5.88 [d, 1H, J = 4.2 Hz, H−C(1′)], 8.09 [s, 1H, H−C(8)]; ^{13}C NMR (100 MHz, CD_3OD/NaOD, HMQC, HMBC, DEPT), δ 14.2 [C(5‴)], 19.0 [C(3″)], 21.1 [C(4‴)], 32.7 [C(2‴, C(3‴))], 40.1 [C(1‴)], 53.1 [C(2″)], 65.0 [d, $^2J_{C,P}$ = 4.6 Hz, C(5′)], 72.2 [C(3′], 76.7 [C(2′)], 85.6 [d, $^3J_{C,P}$ = 8.3 Hz, C(4′)], 90.0 [C(1′)], 119.8 [C(5)], 137.3 [C(8)], 153.0 [C(4)], 161.2 [C(2)], 169.8 [C(6)], 177.1 [C(1″)].

*(S)-N^2-((1-(N-Pentylamino)carbonyl)ethyl)guanosine 5′-monophosphate, (S)-**17***: TOF-MS, m/z 503.1657 (found), m/z 503.1661 (calcd for $[C_{18}H_{28}N_6O_9P]^-$); ^{1}H NMR (400 MHz, CD_3OD/NaOD, COSY), δ 0.82 [t, 3H, J = 7.0 Hz, H−C(5‴), 1.24 [m, 4H, H−C(3‴), H−C(4‴)], 1.39 [d, 3H, J = 7.1 Hz, H−C(3″)], 1.46 [m, 2H, H−C(2‴)], 3.17 [t, 2H, J = 7.0 Hz, H−C(1‴)], 3.96−4.05 [m, 1H, H−C(5′$_a$)], 4.05−4.12 [m, 1H, H−C(5′$_b$)], 4.16 [m, 1H, H−C(4′)], 4.40 [pt, 1H, J = 5.0 Hz, H−C(3′)], 4.46−4.54 [m, 2H, H−C(2′), H−C(2″)], 5.89 [d, 1H, J = 4.2 Hz, H−C(1′)], 8.11 [s, 1H, H−C(8)]; ^{13}C NMR (100 MHz, CD_3OD/NaOD, HSQC, HMBC), δ 14.4 [C(5‴)], 19.0 [C(3″)], 23.4 [C(4‴)], 30.1 [C(3‴)], 30.1 [C(2‴)], 40.3 [C(1‴)], 52.7 [C(2″)], 64.9 [d, $^2J_{C,P}$ = 3.8 Hz, C(5′)], 72.1 [C(3′], 76.9 [C(2′)], 85.6 [d, $^3J_{C,P}$ = 8.2 Hz, C(4′)], 89.7 [C(1′)], 119.5 [C(5)], 136.9 [C(8)], 153.0 [C(4)], 161.1 [C(2)], 169.8 [C(6)], 177.7 [C(1″)].

*(R)-N^2-((1-(N-Hexylamino)carbonyl)ethyl)guanosine 5′-monophosphate, (R)-**18***: TOF-MS, m/z 517.1818 (found), m/z 517.1817 (calcd for $[C_{19}H_{30}N_6O_9P]^-$); ^{1}H NMR (500 MHz, CD_3OD/NaOD, COSY), δ 0.82 [t, 3H, J = 6.8 Hz, H−C(6‴), 1.21 [m, 6H, H−C(3‴), H−C(4‴), H−C(5‴)], 1.39 [d, 3H, J = 7.1 Hz, H−C(3″)], 1.45

[m, 2H, H−C(2‴)], 3.17 [m, 2H, H−C(1‴)], 3.97−4.04 [m, 1H, H−C(5′$_a$)], 4.06−4.12 [m, 1H, H−C(5′$_b$)], 4.14 [m, 1H, H−C(4′)], 4.40 [pt, 1H, J = 5.3 Hz, H−C(3′)], 4.43 [q, 1H, J = 7.1 Hz, H−C(2″)], 4.55 [pt, 1H, J = 4.5 Hz, H−C(2′)], 5.84 [d, 1H, J = 4.0 Hz, H−C(1′)], 8.02 [s, 1H, H−C(8)]; ^{13}C NMR (125 MHz, CD_3OD/NaOD, HMQC, HMBC, DEPT), δ 14.5 [C(6‴)], 19.0 [C(3″)], 23.7 [C(5‴)], 27.7 [C(4‴)], 30.5 [C(3‴)], 32.7 [C(2‴)], 40.5 [C(1‴)], 53.1 [C(2″)], 65.5 [d, $^2J_{C,P}$ = 4.4 Hz, C(5′)], 72.5 [C(3′], 77.2 [C(2′)], 85.8 [d, $^3J_{C,P}$ = 8.0 Hz, C(4′)], 90.8 [C(1′)], 119.8 [C(5)], 137.2 [C(8)], 153.1 [C(4)], 161.3 [C(2)], 170.0 [C(6)], 178.1 [C(1″)].

*(S)-N^2-((1-(N-Hexylamino)carbonyl)ethyl)guanosine 5′-monophosphate, (S)-**18***: TOF-MS, m/z 517.1823 (found), m/z 517.1817 (calcd for $[C_{19}H_{30}N_6O_9P]^-$); ^{1}H NMR (400 MHz, CD_3OD/NaOD, COSY), δ 0.84 [t, 3H, J = 6.7 Hz, H−C(6‴), 1.22 [m, 6H, H−C(3‴), H−C(4‴), H−C(5‴)], 1.38 [d, 3H, J = 7.1 Hz, H−C(3″)], 1.44 [m, 2H, H−C(2‴)], 3.16 [t, J = 7.1 Hz, 2H, H−C(1‴)], 3.98−4.05 [m, 1H, H−C(5′$_a$)], 4.05−4.12 [m, 1H, H−C(5′$_b$)], 4.17 [m, 1H, H−C-(4′)], 4.42 [t, 1H, J = 5.0 Hz, H−C(3′)], 4.49 [q, 1H, J = 7.1 Hz, H−C(2″)], 4.52 [t, 1H, J = 4.7 Hz, H−C(2′)], 5.90 [d, 1H, J = 4.2 Hz, H−C(1′)], 8.13 [s, 1H, H−C(8)]; ^{13}C NMR (100 MHz, CD_3OD/NaOD, HMQC, HMBC, DEPT), δ 14.4 [C(6‴)], 18.9 [C(3″)], 23.6 [C(5‴)], 27.6 [C(4‴)], 30.4 [C(3‴)], 32.7 [C(2‴)], 40.3 [C(1‴)], 52.7 [C(2″)], 64.8 [d, $^2J_{C,P}$ = 4.7 Hz, C(5′)], 72.0 [C(3′], 76.8 [C(2′)], 85.6 [d, $^3J_{C,P}$ = 8.5 Hz, C(4′)], 89.5 [C(1′)], 119.5 [C(5)], 137.0 [C(8)], 153.0 [C(4)], 161.1 [C(2)], 169.8 [C(6)], 177.7 [C(1″)].

*(R)-N^2-((1-(N-Furfurylamino)carbonyl)ethyl)guanosine 5′-monophosphate, (R)-**19***: TOF-MS, m/z 513.1166 (found), m/z 513.1141 (calcd for $[C_{18}H_{22}N_6O_{10}P]^-$); ^{1}H NMR (500 MHz, CD_3OD/NaOD, COSY), δ 1.40 [d, 3H, J = 7.1 Hz, H−C(3′)], 3.97−4.04 [m, 1H, H−C(5′$_a$)], 3.98−4.16 [m, 2H, H−C(4′), H−C(5′$_a$)], 4.30−4.43 [m, 3H, H−C(3′), H−C(1‴)], 4.48 [q, 1H, J = 7.1 Hz, H−C(2″)], 4.55 [pt, 1H, J = 4.6 Hz, H−C(3′)], 5.82 [d, 1H, J = 4.0 Hz, H−C(1)], 6.15 [d, 1H, J = 3.2 Hz, H−C(3‴)], 6.25 [dd, 1H, J = 1.9, 3.2 Hz, H−C(4‴)], 7.32 [d, 1H, J = 1.9 Hz, H−C(5‴)], 8.01 [s, 1H, H−C-(8)]; ^{13}C NMR (125 MHz, CD_3OD/NaOD, HMQC, HMBC, DEPT), δ 19.0 [C(3″)], 37.3 [C(1‴)], 53.0 [C(2″)], 65.6 [d, $^2J_{C,P}$ = 4.3 Hz, C(5′)], 72.5 [C(3′)], 77.2 [C(2′)], 85.8 [d, $^3J_{C,P}$ = 8.2 Hz, C(4′)], 91.0 [C(1′)], 108.0 [C(3‴)], 111.5 [C(4‴)], 119.9 [C(5)], 137.3 [C(8)], 143.3 [C(5‴)], 153.1 [C(4)], 153.3 [C(2‴)], 161.3 [C(2)], 169.9 [C(6)], 178.2 [C(1″)].

*(S)-N^2-((1-(N-Furfurylamino)carbonyl)ethyl)guanosine 5′-monophosphate, (S)-**19***: TOF-MS, m/z 513.1147 (found), m/z 513.1141 (calcd for $[C_{18}H_{22}N_6O_{10}P]^-$); ^{1}H NMR (400 MHz, CD_3OD/NaOD, COSY), δ 1.40 [d, 3H, J = 7.1 Hz, H−C(3′)], 3.98−4.06 [m, 1H, H−C(5′$_a$)], 4.06−4.12 [m, 1H, H−C(5′$_b$)], 4.17 [m, 1H, H−C(4′)], 4.36 [d, 2H, J = 2.0 Hz, H−C(1‴)], 4.43 [pt, 1H, J = 5.0 Hz, H−C(3′)], 4.48−4.56 [m, 2H, H−C(3′), H−C(2″)], 5.90 [d, 1H, J = 4.2 Hz, H−C(1)], 6.14 [d, 1H, J = 3.2 Hz, H−C(3‴)], 6.25 [dd, 1H, J = 1.9, 3.2 Hz, H−C(4‴)], 7.32 [d, 1H, J = 1.9 Hz, H−C(5‴)], 8.14 [s, 1H, H−C(8)]; ^{13}C NMR (100 MHz, CD_3OD/NaOD, HMQC, HMBC, DEPT), δ 18.8 [C(3″)], 37.2 [C(1‴)], 52.7 [C(2″)], 64.7 [d, $^2J_{C,P}$ = 4.6 Hz, C(5′)], 71.9 [C(3′)], 76.7 [C(2′)], 85.6 [d, $^3J_{C,P}$ = 8.4 Hz, C(4′)], 89.6 [C(1′)], 107.8 [C(3‴)], 111.3 [C(4‴)], 119.6 [C(5)], 137.0 [C(8)], 143.1 [C(5‴)], 153.0 [C(4)], 153.2 [C(2‴)], 161.1 [C(2)], 169.8 [C(6)], 177.8 [C(1″)].

Synthesis of N^2-Acylguanosine 5′-monophosphates 7, 20, and 21. For the synthesis of N^2-acylguanosine 5′-monophosphates, guanosine was acylated and, then, phosphorylated following protocols reported earlier.[14,18]

Synthesis of Acylated Guanosines. A mixture of vacuum-dried guanosine hydrate (2.5 mmol), dichloromethane (50 mL), and pyridine (12.5 mL) was cooled to 0 °C, while being constantly stirred, under an atmosphere of argon. Then trimethylsilyl chloride (22.5 mmol) was carefully added, and, after removal of the ice bath, the reaction mixture was stirred for 2 h at room temperature. The homogeneous solution was again cooled to 0 °C, the acetyl chloride, furoyl chloride, or (*S*)-*O*-acetyllactoyl chloride (2.75 mmol each), respectively, was carefully added, and the solution was stirred for 3 h at the same temperature. Thereafter, methanol (10 mL) was added, stirring was continued overnight at room temperature to achieve complete desilylation, and, after separation of the solvent under vacuum, the crude target compounds were obtained as viscous oils.

For N^2-acetylguanosine, methanol (50 mL) was added to the oily residue and, after stirring for 1 h at 0 °C, the target compound was obtained as a white precipitate (86% in yield), which was filtered, washed with methanol, and dried under vacuum. Spectroscopic data were found to be well in accordance with those reported in the literature.[18]

N^2-acetylguanosine: TOF-MS, m/z 324.0947 (found), m/z 324.0950 (calcd for $[C_{12}H_{14}N_5O_6]^-$); ^{1}H NMR (400 MHz, d_6-DMSO, COSY), δ 2.18 [s, 3H, H−C(2″)], 3.54 [dd, 1H, J = 4.1, 12.0 Hz, H−C(5′$_a$)], 3.60−3.67 [dd, 1H, J = 4.1, 12.0 Hz, H−C(5′$_b$)], 3.90 [q, 1H, J = 4.0 Hz, H−C(4′)], 4.14 [t, 1H, J = 4.3 Hz, H−C(2′)], 4.43 [t, 1H, J = 5.3 Hz, H−C(3′)], 4.90−5.64 [3 × bs, 3H, OH−C(3′), OH−C(4′), OH−C-(5′)], 5.80 [d, 1H, J = 5.7 Hz, H−C(1′)], 8.27 [s, 1H, H−C(8)], 11.78 [s, 1H, H−N(2)], 12.05 [s, 1H, H−N(1)]; ^{13}C NMR (100 MHz, d_6-DMSO, HMQC, HMBC, DEPT), δ 23.8 [C(2″)], 61.1 [C(5′)], 70.2 [C(3′)], 74.0 [C(2′)], 85.3 [C(4′)], 86.7 [C(1′)], 120.2 [C(5)], 137.7 [C(8)], 148.0 [C(4)], 148.8 [C(2)], 154.9 [C(6)], 173.5 [C(1″)].

For the preparation of N^2-furoylguanosine, the oily residue was mixed with water (50 mL), and then residues of free pyridine were distilled off. After this process had been repeated three times, the target compound was precipitated upon addition of ice-cold water (50 mL) and, after filtration, washing, and vacuum-drying, was obtained as an amorphous white powder (65% in yield).

N^2-Furoylguanosine: TOF-MS, m/z 376.0905 (found), m/z 376.0899 (calcd for $[C_{15}H_{14}N_5O_7]^-$); ^{1}H NMR (400 MHz, d_6-DMSO, COSY), δ 3.55 [dd, 1H, J = 3.6 Hz, 11.5 Hz, H−C(5′$_a$)], 3.67 [dd, 1H, J = 3.6 Hz, 11.5 Hz, H−C(5′$_b$)], 3.92 [m, 1H, H−C(4′)], 4.13 [t, 1H, J = 4.3 Hz, H−C(2′)], 4.46 [t, 1H, J = 5.3 Hz, H−C(3′)], 4.96−5.56 [3 × bs, 3H, OH−C(3′), OH−C(4′), OH−C(5′)], 5.80 [d, 1H, J = 6.0 Hz, H−C(1′)], 6.77 [dd, 1H, J = 1.6, 3.6 Hz, H−C(4″)], 7.79 [d, 1H, J = 3.6 Hz, H−C(3″)], 8.07 [d, 1H, J = 1.6 Hz, H−C(5″)], 8.30 [s, 1H, H−C(8)], 11.89 [s, 1H, H−N(2)], 12.12 [s, 1H, H−N(1)]; ^{13}C NMR (100 MHz, d_6-DMSO, HMQC, HMBC, DEPT), δ 61.2 [C(5′)], 70.3 [C(3′)], 74.0 [C(2′)], 85.4 [C(4′)], 86.3 [C(1′)], 112.6 [C(4″)], 118.2 [C(3″)], 120.4 [C(5)], 137.7 [C(8)], 145.1 [C(4)], 148.0 [C(4)], 148.2 [C(2)], 149.0 [C(5″)],155.5 [C(6)], 158.6 [C(1″)].

For the purification of N^2-(*S*)-*O*-acetyllactoylguanosine, the oily residue was mixed with water (50 mL) and then residues of free pyridine were distilled off. After this process had been repeated three times, the residue was taken up with water (30 mL) and, then, the title compound was isolated by means of RP-MPLC. Lyophilization of the main fraction showing absorbance at λ = 260 nm afforded N^2-(*S*)-O-acetyllactoylguanosine as an amorphous white powder (59% in yield).

N^2-(*S*)-*O*-acetyllactoylguanosine: TOF-MS, m/z 396.1153 (found), m/z 396.1161 (calcd for $[C_{15}H_{18}N_5O_8]^-$); ^{1}H NMR (400 MHz, d_6-DMSO, COSY), δ 1.45 [d, 3H, J = 6.9 Hz, H−C(3″)], 2.10 [s, 3H, H−C(5″)], 3.49−3.70 [m, 2H, H−C(5′)], 3.91 [q, 1H, J = 3.9 Hz, H−C(4′)], 4.13 [m, 1H, H−C(3′)], 4.44 [dd, 1H, J = 4.9, 9.8 Hz, H−C(2′)], 5.06 [t, 1H, J = 5.2 Hz, OH−C(5′)], 5.11 [q, 1H, J = 6.9 Hz, H−C(2″)], 5.20 [d, 1H, J = 3.1 Hz, OH−C(3′)], 5.49 [bd, 1H, J = 5.3 Hz, OH−C(4′)], 5.82 [d, 1H, J = 5.7 Hz, H−C(1′)], 8.30 [s, 1H, H−C(8)], 11.77 [s, 1H, H−N(2)], 12.02 [s, 1H, H−N(1)]; ^{13}C NMR (100 MHz, d_6-DMSO, HMQC, HMBC, DEPT), δ 16.8 [C(5″)], 20.3 [C(3″)], 61.0 [C(5′)], 69.4 [C(2″)], 70.0 [C(3′)], 74.0 [C(2′)], 85.1 [C(4′)], 86.4 [C(1′)], 120.3 [C(5)], 137.7 [C(8)], 147.3 [C(4)], 148.5 [C(2)], 154.7 [C(6)], 171.8 [C(4″)], 173.5 [C(1″)].

Phosphorylation of Acylated Guanosines. A mixture of the acylated guanosine (1 mmol) and triethyl phosphate (2.5 mL) was stirred at 0 °C under argon. Carefully, phosphoroxychloride (6 mmol) was added, the resulting mixture was stirred for 4 h being constantly cooled with an ice/water bath, and the reaction progress was followed by means of analytical RP-HPLC. After 4 h, the reaction was stopped by the addition of ice (10 g), the pH value was adjusted to 3.0 using aqueous sodium hydroxide (3 mol/L), and the target compound was purified by means of RP-MPLC, followed by preparative RP-HPLC. The fractions showing UV absorbance at λ = 260 nm were collected, separated from solvent under vacuum, and lyophilized twice to yield the corresponding N^2-acylated guanosine 5′-monophosphates **7**, **20**, and **21** as amorphous white powders with purity of >95% (HPLC, ^{1}H NMR).

N^2-Acetylguanosine 5′-monophosphate, ***7****:* TOF-MS, *m/z* 404.0605 (found), *m/z* 404.0613 (calcd for $[C_{12}H_{15}N_5O_9P]^-$); ^{1}H NMR (400 MHz, D_2O, COSY), δ 2.29 [s, 3H, H−C(2″)], 4.10−4.18 [m, 1H, H−C(5′a)], 3.97−4.10 [m, 1H, H−C(5b′)], 4.36 [m, 1H, H−C(4′)], 4.51 [dd, 1H, *J* = 3.3, 5.1 Hz, H−C(3′)], 4.72 [pt, 1H, *J* = 5.4 Hz, H−C(2′)], 6.01 [d, 1H, *J* = 6.2 Hz, H−C(1′)], 8.26 [s, 1H, H−C(8)]; ^{13}C NMR (100 MHz, D_2O, HMQC, HMBC, DEPT), δ 26.3 [C(2″)], 67.4 [d, $^2J_{C,P}$ = 4.8 Hz, C(5′)], 73.4 [C(3′)], 76.4 [C(2′)], 87.1 [d, $^3J_{C,P}$ = 8.7 Hz, C(4′)], 90.6 [C(1′)], 123.0 [C(5)], 142.9 [C(8)], 150.6 [C(4)], 152.4 [C(2)], 160.3 [C(6)], 178.3 [C(1″)].

N^2-Furoylguanosine 5′-monophosphate, ***20****:* TOF-MS, *m/z* 456.0577 (found), *m/z* 456.0562 (calcd for $[C_{15}H_{15}N_5O_{10}P]^-$); ^{1}H NMR (400 MHz, CD_3OD/NaOD, COSY), δ 3.96−4.05 [m, 1H, H−C(5′$_a$)], 4.05−4.14 [m, 1H, H−C(5′$_b$)], 4.22 [m, 1H, H−C(4′)], 4.37 [pt, 1H, *J* = 5.0 Hz, H−C(3′)], 4.56 [pt, 1H, *J* = 4.9 Hz, H−C(2′)], 5.96 [d, 1H, *J* = 4.5 Hz, H−C(1′)], 6.55 [dd, 1H, *J* = 1.8, 3.4 Hz, H−C(4″)], 7.17 [d, 1H, *J* = 3.4 Hz, H−C(3″)], 7.64 [d, 1H, *J* = 1.8 Hz, H−C(5″)], 8.33 [s, 1H, H−C(8)]; ^{13}C NMR (100 MHz, CD_3OD/NaOD, HMQC, HMBC), δ 64.6 [d, C(5′)], 71.5 [C(3′)], 76.7 [C(2′)], 85.8 [d, C(4′)], 89.5 [C(1′)], 112.5 [C(3″)], 114.5 [C(4″)], 120.5 [C(5)], 139.0 [C(8)], 145.6 [C(5″)], 152.8 [C(4)], 153.2 [C(2″)], 159.3 [C(2)], 162.7 [C(6)], 169.9 [C(1″)].

N^2-(S)-O-Acetyllactoylguanosine 5′-monophosphate, ***21****:* TOF-MS, *m/z* 476.0811 (found), *m/z* 476.0824 (calcd for $[C_{15}H_{19}N_5O_{11}P]^-$); ^{1}H NMR (400 MHz, CD_3OD, COSY), δ 1.54 [d, 3H, *J* = 7.0 Hz, H−C(3″)], 2.14 [s, 3H, H−C(5″)], 4.11−4.29 [m, 3H, H−C(4′), H−C(5′)], 4.51 [dd, 1H, *J* = 3.0, 5.0 Hz, 1H, H−C(3′)], 4.72 [pt, 1H, *J* = 5.4 Hz, H−C(2′)], 5.13 [q, 1H, *J* = 6.9 Hz, H−C(2″)], 5.95 [d, 1H, *J* = 6.0 Hz, H−C(1′)], 8.22 [s, 1H, H−C(8)]; ^{13}C NMR (100 MHz, CD_3OD, HMQC, HMBC), δ 17.4 [C(5″)], 20.5 [C(3″)], 66.8 [d, $^2J_{C,P}$ = 5.2 Hz, C(5′)], 71.5 [C(2″)], 72.2 [C(3′)], 75.3 [C(2′)], 85.3 [d, $^3J_{C,P}$ = 8.8 Hz, C(4′)], 90.1 [C(1′)], 121.7 [C(5)], 140.5 [C(8)], 150.4 [C(4)], 156.3 [C(2)], 157.3 [C(6)], 172.1 [C(4″)], 175.4 [C(1″)].

Preparation of N^2-(*S*)-Lactoylguanosine 5′-Monophosphate (*S*)-22. The deacylation procedure reported in the literature[19] was slightly modified: A solution of N^2-(*S*)-*O*-acetyllactoylguanosine 5′-monophosphate (0.1 mmol) and K_2CO_3 (2 mL, 0.25 mol/L in water/methanol (1:1, v/v)) was stirred for 20 min at room temperature. Thereafter, the pH value was adjusted to 3.5 by the addition of hydrochloric acid (1 mol/L), and the mixture was purified by means of preparative RP-HPLC. The main reaction product, showing UV absorbance at λ = 260 nm, was collected, separated from solvent under vacuum, and lyophilized twice to yield the title compound ((*S*)-**22**) as an amorphous, white residue with a purity of >95% (HPLC, ^{1}H NMR).

*N^2-(S)-Lactoylguanosine 5′-monophosphate, (S)-****22****:* TOF-MS, *m/z* 434.0716 (found), *m/z* 434.0719 (calcd for $[C_{13}H_{17}N_5O_{10}P]^-$); ^{1}H NMR (400 MHz, D_2O, COSY), δ 1.50 [d, 3H, *J* = 7.0 Hz, H−C(3″)], 4.03−4.20 [m, 2H, H−C(5′)], 4.36 [m, 1H, H−C(4′)], 4.49 [m, 1H, H−C(3′)], 4.53 [q, 1H, H−C(2″)], 4.72 [pt, 1H, *J* = 5.6 Hz, H−C(2′)], 6.03 [d, 1H, *J* = 6.0 Hz, H−C(1′)], 8.33 [s, 1H, H−C(8)]; ^{13}C NMR (100 MHz, D_2O, HMQC, HMBC), δ 22.3 [C(3‴)], 67.5 [d, $^2J_{C,P}$ = 5.0 Hz, C(5′)], 70.8 [C(2″)], 73.5 [C(3′)], 76.9 [C(2′)], 87.1 [d, $^3J_{C,P}$ = 8.8 Hz, C(4′)], 90.5 [C(1′)], 123.1 [C(5)], 142.7 [C(8)], 150.3 [C(4)], 152.3 [C(2)], 160.3 [C(6)], 181.3 [C(1″)].

Functional hT1R1/rT1R3 Umami Receptor Experiments. Functional experiments were carried out in the human embryonic kidney cell line PEAK^{Rapid} (American Type Culture Collection, Manassas, VA) stably expressing the human umami receptor subunit T1R1 and the G protein subunit mGα15. Human T1R1 was constructed via amplification of all exons from fungiform papilla cDNA by recombinant polymerase chain reactions (PCR). Cells were cultured under regular conditions at 37 °C, 5% CO_2, and 95% humidity in Dulbecco's Modified Eagle Medium (D-MEM) High Glucose GlutaMAX (Invitrogen, Karlsruhe, Germany) supplied with fetal calf serum (10%, tetracycline-free), penicillin G (10000 units/mL), and streptomycin (10 mg/mL) and selected for stable expression of hT1R1 and mGα15 with G418 (50 μg/mL), zeocin (100 μg/μL), hygromycin (100 μg/mL, all Invitrogen) and puromycin (1.0 μg/mL, Sigma-Aldrich, Schnelldorf, Germany).

For functional experiments, cells were seeded into 96-well plates (Greiner Bio-One, Frickenhausen, Germany) coated with poly-D-lysine (10 μg/mL) to improve cell adhesion. To enable expression of a functional umami receptor, cells were transfected with rat T1R3 (cloned from rat circumvallate papilla cDNA, a gift from H. Schmale, University Medical Center Hamburg-Eppendorf, Hamburg, Germany) using Lipofectamine 2000 (Invitrogen) in serum-free D-MEM Low Glucose GlutaMAX (Invitrogen). After transfection, cells were cultured in D-MEM Low Glucose GlutaMAX with dialyzed, tetracycline-free FCS for about 24 h. Prior to the experiment, cells were incubated for 1 h with Fluo4-AM (2 μmol/L, Molecular Probes) in serum-free D-MEM Low Glucose GlutaMAX containing 2.5 mM probenecide to block leakage of the fluorescent dye. Cells were washed with a bath solution (130 mmol/L NaCl, 5 mmol/L KCl, 10 mmol/L HEPES, 2 mmol/L $CaCl_2$, 10 mmol/L glucose, pH 7.4) three times and incubated at room temperature for ~40 min to allow complete de-esterification of the fluorescent dye. Test substances were dissolved in the bath solution. For coapplication experiments, the individual substances were premixed prior to application. Intracellular calcium levels were recorded during automated application of test solutions using a fluorometric imaging plate reader (FLIPR^{384}, Molecular Devices, Munich, Germany). To control for cell number and vitality, isoproterenol was applied subsequent to test compounds. Cells transfected with empty vector receiving equal stimuli served as control for unspecific responses of the cellular background. Data were collected from three independent experiments carried out in quadruplicates and duplicates (concentration−response), respectively. Raw fluorescence signals of hT1R1/rT1R3-expressing cells were reduced by fluorescence signals of mock-transfected cells (software FLIPR^{384}, Molecular Devices) and normalized to baseline fluorescence ($\Delta F/F$, SigmaPlot 9.01, Systat Software GmbH, Erkrath, Germany). For the calculation of concentration−response, $\Delta F/F$ values were plotted half-logarithmically against test substance concentrations. Half-maximal effective agonist concentrations (EC_{50}) were calculated by using nonlinear regression to the sigmoidal function $f(x)$ = min + (max − min/1 + $[x/EC_{50}]^{Hillslope}$).

Fluorescence signals after coapplication of test compounds with L-glutamic acid were analyzed for statistical significance compared to sole application of L-glutamic acid using one-way analysis of variance followed by a Tukey's post hoc means comparison test with 5% α-risk level (GraphPad Prism 4.03, GraphPad Software, Inc., La Jolla, CA).

Reversed Phase Medium-Pressure Liquid Chromatography (RP-MPLC). Medium-pressure liquid chromatography was performed on a Sepacore preparative chromatography system (Büchi, Flawil, Switzerland) consisting of two pumps (C-605), a pump manager (C-615), a fraction collector (C-660), a manual injection port equipped with a 20 mL loop, and an UV detector (C-635) monitoring the effluent at 260 nm. Chromatography was performed on a 150 × 40 mm i.d. polypropylene cartridge (Büchi) filled with a slurry of LiChroprep,

25−40 μm, RP-18 material (Merck) as stationary phase and using a gradient of 1% aqueous formic acid (solvent A) and methanol (solvent B) as the mobile phase (flow rate = 40 mL/min). Starting with an isocratic elution with 100% A for 5 min, the content of solvent B was increased linearly to 100% within 55 min. The fractions showing UV absorption at 260 nm were collected individually, freed from solvent under vacuum.

High-Performance Liquid Chromatography (HPLC). The analytical HPLC system (Jasco, Gross-Umstadt, Germany) consisted of a PU-2080 Plus pump, a DG-2080-53 degasser, an LG-2080-02 gradient unit, an AS-2055 Plus autosampler with a 100 μL loop, and an MD-2010 Plus detector. Separations in analytical scale were performed on a C18 column, 250 × 4.6 mm i.d., 5 μm (Trentec, Rutesheim, Germany). Chromatography was performed with a mixture of 1% formic acid in water (solvent A) and acetonitrile (solvent B) at a flow rate of 1 mL/min, monitoring the effluent at 260 nm, starting the separation with an isocratic step of 0% B for 5 min, afterward increasing the content of B to 5% in 10 min and to 10% B within further 5 min. Thereafter, a linear gradient to 30% B in 10 min was applied before finally increasing the content of B to 100% within 10 min.

The HPLC apparatus (Jasco, Gross-Umstadt, Germany) for preparative liquid chromatography consisted of two PU-2087 pumps, a Degasys DG-1310 online degasser (Uniflows Co., Tokyo, Japan), a 1000 μL gradient mixer, a 7725 i injection valve (Rheodyne, Bensheim, Germany), and an MD-2010 Plus detector. Chromatographic separations in preparative scale were conducted on a Microsorb-MV C18 column, 250 × 21.2 mm i.d., 5 μm (Varian, Darmstadt, Germany), operating at a flow rate of 15 mL/min. Using 1% formic acid in water (solvent A) and methanol (solvent B) as solvent, chromatography started with 5% B for 5 min followed by a linear gradient to 100% B within 30 min, and finally maintaining 100% B for 2 min.

LC−Time-of-Flight Mass Spectrometry (LC-TOF-MS). Mass spectra of the target compounds were measured on a Bruker Micro-TOF-Q (Bruker Daltonics, Bremen, Germany) mass spectrometer with flow injection referenced on sodium formate. Data processing was performed by using Daltonics DataAnalysis software (version 3.4, Bruker Daltonics).

Liquid Chromatography−Mass Spectrometry (LC-MS). Electrospray ionization (ESI) spectra were acquired on an API 3200 type LC-MS/MS system (AB Sciex Instruments, Darmstadt, Germany) coupled to an Agilent 1100 HPLC system operating at a flow rate of 200 μL/min with direct loop injection of the sample (2−20 μL). The spray voltage was set at −4500 V in ESI^- mode. Zero grade air served as nebulizer gas (35 psi) and as turbo gas (350 °C) for solvent drying (45 psi). Nitrogen served as curtain (20 psi) and collision gas (4.5×10^{-5} Torr). Both quadrupoles were set at unit resolution. The declustering potential was set at −10 to −40 V in ESI^- mode. The mass spectrometer was operated in the full scan mode monitoring positive and negative ions.

Nuclear Magnetic Resonance Spectroscopy (NMR). The ^1H, ^{13}C, COSY, DEPT, HMQC, HSQC, and HMBC spectroscopic experiments were performed on either a DRX-400 or a 500 MHz Avance III NMR spectrometer from Bruker (Rheinstetten, Germany). Samples were dissolved in d_6-dimethyl sulfoxide (d_6-DMSO), deuterium oxide (D_2O), deuterated methanol (CD_3OD), or a mixture (75:1, v/v) of CD_3OD and sodium deuteroxide (40% w/w solution in D_2O). Both d_6-DMSO and CD_3OD contained tetramethylsilane (TMS) as reference. When deuterium oxide was used as solvent, 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (TMSP) was added as reference. Whereas data processing was performed using Topspin version 1.3 (Bruker), the individual data interpretation was done with MestReNova 5.1.0-2940 (Mestrelab Research S.L., Santiago de Compostela, Spain).

RESULTS AND DISCUSSION

Recent application of a sensomics approach led to the identification of (*S*)-N^2-(1-carboxyethyl)guanosine 5′-monophosphate, (*S*)-**9** (Figure 1), as a previously not reported umami-enhancing nucleotide in yeast extract.[15] Model studies on its formation revealed that (*S*)-**9** is generated together with its nearly taste-inactive (*R*)-configured stereoisomer by a Maillard-type reaction of 5′-GMP and 1,3-dihydroxyacetone or glyceraldehyde, respectively. In addition, amides of (*R*)/(*S*)-**9** were produced when the reaction was performed in the presence of an amine; for example, the umami-enhancing amide (*S*)-**10** was obtained besides the sensorially inactive (*R*)-isomer in the presence of *n*-propylamine.[16] As the efficiency of this Maillard-type synthesis to produce such amides of **9** was found to be limited by the low solubility and reactivity of long-chain alkyl amines as well as by the tedious separation of the diastereomeric amides formed,[16] a versatile synthesis for the (*R*)- and (*S*)-configured amides of N^2-carboxyalkylated guanosine 5′-monophosphate (**9**) was developed following the strategy depicted in Figure 2.

Synthesis of N^2-(1-Carboxyethyl)guanosine 5′-Monophosphates (*R*)-9 and (*S*)-9. To be used as the starting material for amidation (Figure 2), an optimized procedure for the preparation of the diastereomers (*R*)- and (*S*)-**9** from **2** and 1,3-dihydroxyacetone was developed. Whereas the recently reported reaction was performed at 40 °C for 10 days,[15] optimizing the reaction conditions led to a quantitative conversion of **2** at 70 °C within 24 h. Purification of the reaction products by RP-MPLC enabled the rapid, baseline-separated gram-scale preparation of (*R*)- and (*S*)-**9** with a purity of >90% (HPLC, ^1H NMR).

Amidation of N^2-(1-Carboxyethyl)guanosine 5′-Monophosphate, (*R*)/(*S*)-9. Preliminary studies revealed that the amidation of **9** was limited due to poor solubility in organic solvents. However, reacting the nucleotide **9** with a 4-fold molar excess of an amine in the presence of the water-soluble coupling agent *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride in aqueous solution was found to be suitable for amide synthesis when the pH value was maintained between 4.7 and 5.0 during the entire reaction time (Figure 2).

As an example, the influence of the reaction time on the formation of (*S*)-**10** from (*S*)-**9** and *n*-propylamine is illustrated in Figure 3. Whereas the starting compound eluted after about 22 min (Figure 3A), the reaction product (*S*)-**10** could be detected at 24 min right after the reactants had been mixed (Figure 3B). After 4 h, a nearly quantitative conversion of the starting material into (*S*)-**10** was observed accompanied by trace amounts of (*R*)-**10** formed upon racemization (Figure 3C). To remove any trace amounts of the amine as well as the coupling agent, the target compound was purified by means of preparative RP-HPLC, followed by rechromatography to yield (*S*)-N^2-((1-(*N*-propylamino)carbonyl)ethyl)guanosine 5′-monophosphate, (*S*)-**10**, in a purity of >98% (HPLC, ^1H NMR). Retention time (HPLC), TOF-MS, ^1H and ^{13}C NMR data were identical to those published recently for (*S*)-**10** isolated from a Maillard reaction system.[16]

Following this straightforward synthetic approach, both (*S*)-**9** and (*R*)-**9** were coupled with a series of amines to give the (*R*)- and (*S*)-configured N^2-(1-alkylamino)carbonylalkyl)guanosine 5′-monophosphates **10**−**19** in yields ranging from 48 to 74% and a purity of >98% (Figure 4).

Synthesis of N^2-Acylguanosine 5′-Monophosphates. To compare the sensory activities of the N^2-(1-alkylamino)-carbonylalkyl)guanosine 5′-monophosphates **10**−**19** to those of N^2-lactoyl- (**22**) and N^2-acetylguanosine 5′-monophosphate (**7**) (Figure 1), reported as umami modulators in the literature,[12,13] N^2-acylguanosine 5′-monophosphates were synthesized by acylation of guanosine, followed by phosphorylation.[14]

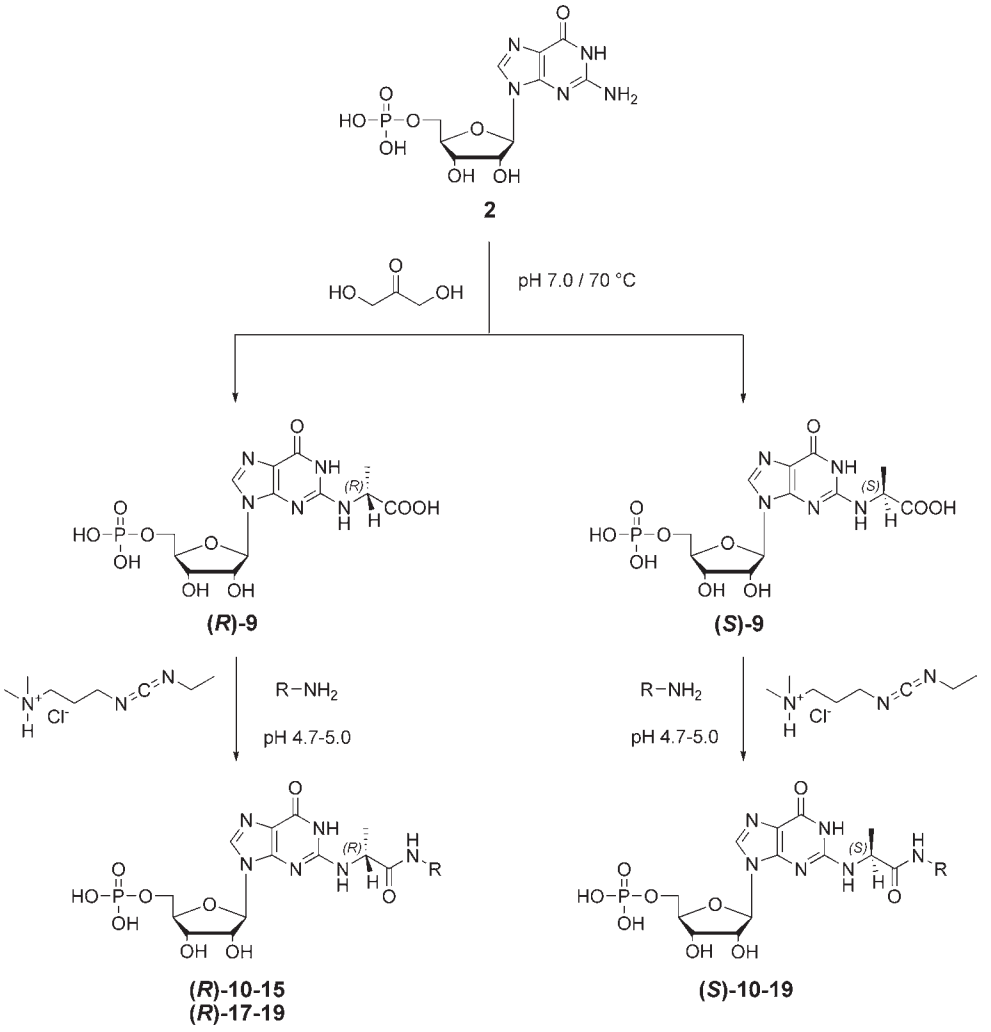


Figure 2. Synthetic sequence leading to the amides (*R*)-**10**−**15**, (*R*)-**17**−**19**, and (*S*)-**10**−**19** starting from 5′-GMP (**2**).

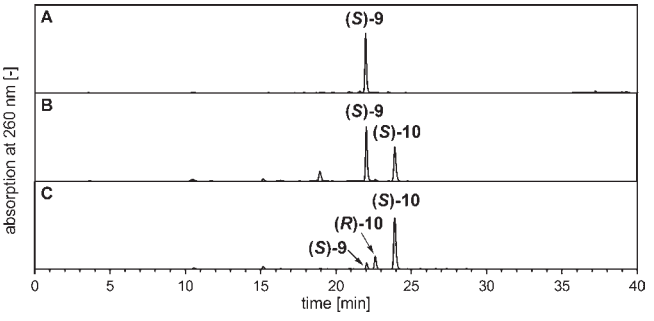
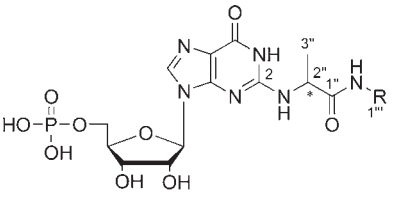


Figure 3. RP-HPLC chromatograms (λ = 260 nm) recorded for purified (*S*)-**9** (A), right after mixing with *n*-propylamine and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (B), and after keeping the mixture for 4 h at pH 4.7−5.0 at room temperature (C), respectively.

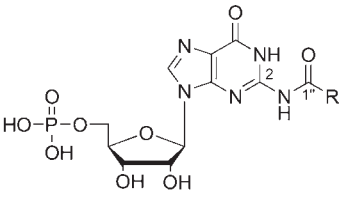
Following the literature protocol for a transient silylation with some modifications,[18] guanosine was first reacted with trimethylsilyl chloride in dichloromethane/pyridine and, then, subjected to a selective *N*-acylation using acetyl chloride, furoyl chloride, and (*S*)-*O*-acetyllactoyl chloride, respectively. After removal of trimethylsilyl protection groups upon methanolysis, the crude mixtures were concentrated, and the target compounds N^2-acetylguanosine and N^2-furoylguanosine were purified by precipitation from methanol or water, whereas N^2-(*S*)-*O*-acetyllactoylguanosine was isolated by means of RP-MPLC, respectively.

After vacuum-drying, the N^2-acylguanosines were subjected to phosphorylation using phosphoroxychloride in triethyl phosphate[11] to give the corresponding N^2-acylguanosine 5′-monophosphates **7**, **20**, and **21** (Figure 4) in yields of 87, 69, and 75%, respectively. Isolation by means of preparative RP-HPLC afforded the target compounds in purity of >95% (HPLC, ^{1}H NMR). To convert N^2-(*S*)-*O*-acetyllactoylguanosine 5′-monophosphate (**21**) into N^2-lactamide **22**, a portion of purified **21** (Figure 4) was saponified in an alkaline methanol/water mixture[19] and, then, purified by means of preparative RP-HPLC.

Human Sensory Studies on Nucleotides. After purity confirmation by means of ^{1}H NMR and HPLC-MS, the individual nucleotides **1**, **2**, **7**, and **9**−**22** were subjected to a preliminary sensory analysis in water. Compounds **1**, **2**, **7**, and **20**−**22**, as well as the (*S*)-configured compounds **10**−**19**, showed an intrinsic umami taste, whereas the corresponding (*R*)-epimers did not (data not shown). To evaluate the umami-enhancing properties of these nucleotides, a paired-choice comparison test was performed using binary mixtures of MSG and 5′-IMP as references.[16,20]



	R
10	-$(CH_2)_2$-CH_3
11	-CH_3
12	-CH_2-CH_3
13	-$CH(CH_3)_2$
14	-$CH(CH_2$-$CH_2)$
15	-$(CH_2)_3$-CH_3
16	-CH_2-$CH((CH_3)_2)$
17	-$(CH_2)_4$-CH_3
18	-$(CH_2)_5$-CH_3
19	-furfuryl



	R
7	-CH_3
20	-furyl
21	-CH(OAc)-CH_3
22	-CH(OH)-CH_3

Figure 4. Chemical structures of N^2-(1-alkylamino)carbonylalkyl)guanosine 5′-monophosphates (**10**−**19**) and N^2-acylguanosine 5′-monophosphates (**7** and **20**−**22**), respectively.

Binary mixtures containing constant levels of MSG and increasing concentrations of 5′-IMP served as references to determine the so-called β values, representing the potency of a test compound to enhance the umami taste of the L-glutamate-containing matrix in relation to 5′-IMP as the reference.[17] As a positive control, the β value was determined for 5′-GMP to be 2.4, being well in line with previously published data.[8,14]

Among the entire series of N^2-(1-alkylamino)carbonylalkyl)-guanosine 5′-monophosphates, the (*S*)-configured isomers were found to have β values ranging from 3.4 to 7.7 strongly depending on the structure of the alkylamide, among which butylamide (*S*)-**15** and isobutylamide (*S*)-**16** showed the highest umami enhancement activities (Table 1). In contrast, the (*R*)-configumers exhibited only a marginal β value of 0.1, thus demonstrating that the (*S*)-configuration at the α-carbon atom of the alanine moiety is a prerequisite for the umami-enhancing activity of these amides as recently found for the carboxylic acid (*S*)-**9**.[16]

Sensory analysis of the N^2-acylguanosine 5′-monophosphates **7** and **20**−**22** revealed significantly lower β values when compared to the N^2-(1-alkylamino)carbonylalkyl)guanosine 5′-monophosphates. The highest β value of 2.7 was found for the furoyl derivative **20**, followed by the acetylated nucleotide **7** (1.9), whereas N^2-(*S*)-*O*-acetyllactoylguanosine 5′-monophosphate (**21**) showed only a marginal β value of 0.2 (Table 1).

Enhancing Effect of Nucleotides on Functionally Expressed T1R1/T1R3 Umami Receptor. Although the candidate receptors for mediating the taste responses to umami substances in humans were identified almost a decade ago,[1,2] only a few studies have combined human psychophysical experiments with functional expression of T1-receptors so far.[21,22] As the human sensory data obtained for the N^2-(1-alkylamino)carbonylalkyl)guanosine 5′-monophosphates implied high stereoselectivity of the umami receptor binding site, the enhancing effect of selected nucleotide derivatives on the L-glutamate-induced response of the functionally expressed T1R1/T1R3 umami receptor was investigated. In human embryonic kidney PEAK^{Rapid} cells that stably express the G protein subunit mGα15, activation of the heteromeric umami receptor combination of T1R1 and T1R3 was coupled to the release of calcium from internal stores that can be detected by fluorescent dyes. Because L-glutamate and nucleotide enhancer are known to interact with the T1R1 subunit of the umami receptor heteromer, our functional expression system is likely to resemble the detection of umami compounds in human taste receptor cells.[7,23] With regard to its sensitivity to L-glutamate, the pharmacological characteristics of our umami functional expression system were well in accordance with previously published in vitro data.[7]

The calcium traces of T1R1/T1R3-expressing cells and mock-transfected cells (control) upon bath application of the test compounds **1**, **2**, (*R*)-**9**, and (*S*)-**9** (0.05 mmol/L each) in the presence or absence of L-glutamic acid (0.5 mmol/L) are depicted in Figure 5A. Being well in agreement with the findings of the psychophysical experiments, compounds **1**, **2**, and (*S*)-**9** enhanced the receptor response on L-glutamate challenge, whereas the (*R*)-epimer of **9** was inactive. Comparison of the responses of T1R1/T1R3-expressing cells to sole application of L-glutamic acid (0.5 mmol/L) and to coapplication with the (*S*)- and (*R*)-N^2-(1-alkylamino)carbonylalkyl)guanosine 5′-monophosphates **9**, **10**, **13**, **14**, **18**, and **19** (0.05 mmol/L) revealed significantly increased receptor responses ($P < 0.05$) in the presence of the (*S*)-configured isomers (Figure 5B). Well in agreement with the human sensory data, none of the (*R*)-isomers enhanced the receptor response to L-glutamate. Also, the tested N^2-acylguanosine 5′-monophosphates **7** and **22** affected the T1R1/T1R3 umami receptor in the presence of L-glutamate; however, only the effect of N^2-acetylguanosine 5′-monophosphate (**7**) was significant (Figure 5B). The lower enhancing activities of these N^2-acylguanosine 5′-monophosphates when compared to those of the (*S*)-N^2-(1-alkylamino)carbonylalkyl)guanosine 5′-monophosphates is well in line with the lower β values found for that class of compounds by means of human sensory studies (Table 1).

To analyze the umami-enhancing properties of the nucleotide derivatives in more detail, we selected the stereoisomers (*R*)-**9**

Table 1. Umami-Enhancing Activity (β Value) of 5′-GMP Derivatives, Related to Inosine 5′-Monophosphate (1)

test nucleotide[a]	β value[b]
inosine 5′-monophosphate (5′-IMP), **1**	1.0
guanosine 5′-monophosphate (5′-GMP), **2**	2.4
(*R*)-N^2-(1-carboxyethyl) 5′-GMP, (*R*)-**9**	0.1[c]
(*S*)-N^2-(1-carboxyethyl) 5′-GMP, (*S*)-**9**	7.0[c]
(*R*)-N^2-((1-(*N*-methylamino)carbonyl)ethyl) 5′-GMP, (*R*)-**11**	0.1
(*S*)-N^2-((1-(*N*-methylamino)carbonyl)ethyl) 5′-GMP, (*S*)-**11**	4.4
(*R*)-N^2-((1-(*N*-ethylamino)carbonyl)ethyl) 5′-GMP, (*R*)-**12**	0.1
(*S*)-N^2-((1-(*N*-ethylamino)carbonyl)ethyl) 5′-GMP, (*S*)-**12**	4.9
(*R*)-N^2-((1-(*N*-propylamino)carbonyl)ethyl) 5′-GMP, (*R*)-**10**	0.1[c]
(*S*)-N^2-((1-(*N*-propylamino)carbonyl)ethyl) 5′-GMP, (*S*)-**10**	6.0[c]
(*R*)-N^2-((1-(*N*-isopropylamino)carbonyl)ethyl) 5′-GMP, (*R*)-**13**	0.1
(*S*)-N^2-((1-(*N*-isopropylamino)carbonyl)ethyl) 5′-GMP, (*S*)-**13**	6.0
(*R*)-N^2-((1-(*N*-cyclopropylamino)carbonyl)ethyl) 5′-GMP, (*R*)-**14**	0.1
(*S*)-N^2-((1-(*N*-cyclopropylamino)carbonyl)ethyl) 5′-GMP, (*S*)-**14**	3.4
(*R*)-N^2-((1-(*N*-butylamino)carbonyl)ethyl) 5′-GMP, (*R*)-**15**	0.1
(*S*)-N^2-((1-(*N*-butylamino)carbonyl)ethyl) 5′-GMP, (*S*)-**15**	7.6
(*S*)-N^2-((1-(*N*-isobutylamino)carbonyl)ethyl) 5′-GMP, (*S*)-**16**	7.6
(*R*)-N^2-((1-(*N*-pentylamino)carbonyl)ethyl) 5′-GMP, (*R*)-**17**	0.1
(*S*)-N^2-((1-(*N*-pentylamino)carbonyl)ethyl) 5′-GMP, (*S*)-**17**	4.6
(*R*)-N^2-((1-(*N*-hexylamino)carbonyl)ethyl) 5′-GMP, (*R*)-**18**	0.1
(*S*)-N^2-((1-(*N*-hexylamino)carbonyl)ethyl) 5′-GMP, (*S*)-**18**	4.0
(*R*)-N^2-((1-(*N*-furfurylamino)carbonyl)ethyl) 5′-GMP, (*R*)-**19**	0.1
(*S*)-N^2-((1-(*N*-furfurylamino)carbonyl)ethyl) 5′-GMP, (*S*)-**19**	3.5
N^2-acetyl 5′-GMP, **7**	1.9
N^2-furoyl 5′-GMP, **20**	2.7
N^2-(*S*)-*O*-acetyllactoyl 5′-GMP, **21**	0.2
N^2-(*S*)-lactoyl 5′-GMP, **22**	0.4

[a] The chemical structures of the nucleotides are displayed in Figures 1 and 4. [b] β values were determined by comparison of the test solution (3 mmol/L MSG + 0.05 mmol/L nucleotide, pH 6.0) with solutions containing MSG (3 mmol/L, pH 6.0) and increasing concentrations of 5′-IMP. [c] Taken from ref 16.

and (*S*)-**9** (0.05 mmol/L each) as representatives to determine their concentration−response relationship on T1R1/T1R3-expressing cells in comparison to **1** and **2**, respectively (Figure 6). We plotted the $\Delta F/F$ ratios of three independent experiments half-logarithmically against the concentration of L-glutamate and calculated the half-maximal effective concentrations (EC_{50}) by nonlinear regression. The presence of the nucleotides induced a shift of the concentration−response functions toward lower levels of L-glutamate, thus demonstrating their receptor enhancement activity. Among these test compounds, the strongest effect was found for (*S*)-**9**, showing a 2 or 12 times lower half-maximal effective concentration (EC_{50} value) of 0.12 mmol/L when compared to L-glutamate in the presence (0.25 mmol/L) or absence (1.43 mmol/L) of 5′-GMP ($P < 0.001$). Again, the (*R*)-isomer was only marginally active and showed an EC_{50} value of 1.05 mmol/L.

Comparing the human sensory data (Table 1) obtained for (*S*)-**9** and the N^2-(1-alkylamino)carbonylalkyl)guanosine 5′-monophosphates (*S*)-**10**, (*S*)-**13**, (*S*)-**14**, (*S*)-**18**, and (*S*)-**19** with their umami receptor enhancing activity demonstrated clear deviations. Although the receptor responses to these nucleotide derivatives were rather similar (Figure 5), the human β values determined for (*S*)-**9** (7.0), (*S*)-**10** (6.0), and (*S*)-**13** (6.0) were

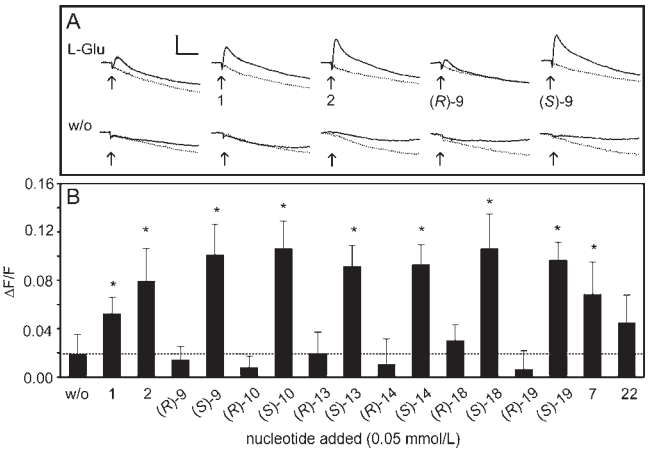


Figure 5. Enhancing effect of nucleotides on the functionally expressed T1R1/T1R3 umami receptor. (A) Calcium traces of T1R1/T1R3-expressing cells (solid lines) and mock-transfected cells (dotted lines) upon bath application (↑) of selected test compounds (0.05 mmol/L) in the presence (0.5 mmol/L, upper row) and absence of L-glutamic acid (lower row), respectively. Scale: *y*, 900 counts; *x*, 2 min. (B) Responses of T1R1/T1R3-expressing cells to sole application of L-glutamic acid (without 0.5 mmol/L) and to coapplication with nucleotides (0.05 mmol/L). Fluorescence signals were reduced by and normalized to background fluorescence ($\Delta F/F$). Significantly increased responses to coapplication versus L-glutamic acid alone are indicated by asterisks ($P < 0.05$).

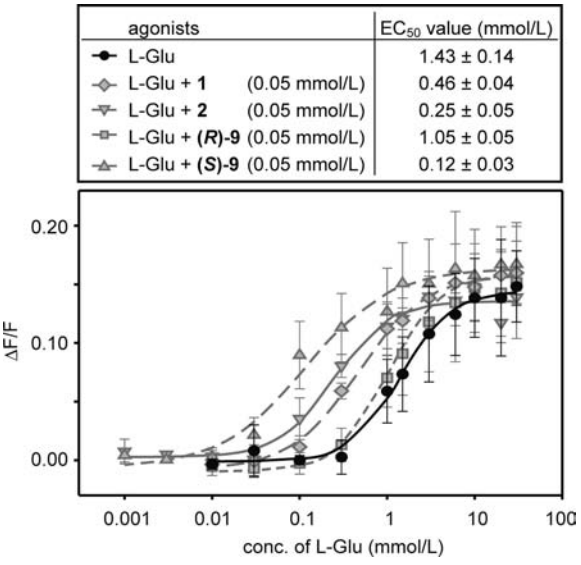


Figure 6. Concentration−response relationships of L-glutamic acid (L-Glu) in the absence (circles, black solid line) and presence of selected nucleotide derivatives (0.05 mmol/L, gray lines) in T1R1/T1R3-expressing cells, $n = 3$. Concentrations for half-maximal receptor activation (EC_{50}) were calculated using SigmaPlot 9.

clearly above those found for (*S*)-**14** (3.4), (*S*)-**18** (4.0), and (*S*)-**19** (3.5). As heterologous functional expression of chimeric receptors consisting of human T1R1 and rodent T1R3 was highly selective for L-glutamate and consistent with psychophysical evidence[1] and the proposed binding site for L-glutamate found to be located in the Venus flytrap domain of the extracellular domain of T1R1,[2] the use of the rodent T1R3 in the

receptor assay experiments is rather unlikely to explain these differences. As recently reported for some food bitter compounds,[24] the observed differences between receptor activation in vitro and umami perception in vivo might appear to account for the increased hydrophobicity of the nucleotides (*S*)-**14**, (*S*)-**18**, and (*S*)-**19** that might be sequestered differently by oral proteins and/or mucosa, affecting the proportion of molecules that are available for receptor activation. Analytical techniques enabling the measurement of the in-mouth retention of taste molecules are urgently required and will have to be developed in the future.

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