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UNDERSTANDING THE MECHANISM OF SWEET TASTE: SYNTHESIS OF TRITIUM LABELED GUANIDINEACETIC ACIDS.

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SUMMARY

Syntheses of tritium labeled guanidineacetic acid sweetener and a tritiated photoaffinty labeling reagent via the catalytic hydrogenation of the dibromo intermediates are described. These labeled compounds were required for the investigation of sweet taste mechanism.

Key Words: Tritium Label, Catalytic Reduction, Photoaffinity Label, Guanidine Sweetener, Antibody Assay, Sweet Taste Mechanism.

INTRODUCTION

The perception of sweet taste is assumed to be initiated by the binding of the sweet stimuli to receptor molecules on the surface of the tongue. Attempts have been made in the past to isolate receptors from bovine, rodent and even primate sources[1]. Isolation of the receptor has been difficult due to the lack of availability of potent agonists or antagonists. Guanidineacetic acids with potencies in excess of 100,000 times that of sucrose have been recently reported by Nofre and Tinti[2].

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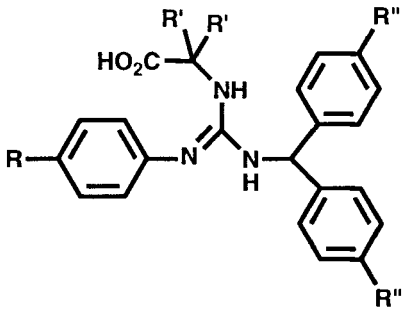
This suggested to us preparation of the necessary molecular probes may now be feasible.

Recently we disclosed the synthesis of photoaffinty labeling guanidines and the *in vivo* effects (nerve response) of irradiation of these reagents on monkeys tongue.[3] The preliminary results indicated that the taste response was inhibited as shown by the nerve response. The double labeling will greatly facilitate experiments designed to identify and isolate specific receptors for these sweet taste



1, R = CN; R' = H; R" = H

2, R = N_3; R' = H; R" = H

3, R = CN; R' = T; R" = H

4, R = CN; R' = H; R" = T

5, R = N_3; R' = H; R" = T

agonists. Tritium labeled ligands offer excellent opportunities to track biochemical preparations with minimal impact on receptor binding. Potent tritium labeled ligands have further utility for competitive binding studies with putative receptors and monoclonal antibody receptor models. The guanidine 1 was chosen for competitive binding assays and the photoaffinty labeling guanidine 2 was chosen for the receptor tagging and isolation studies. We report here the successful syntheses of tritium labeled guanidineacetic acids 3, 4, and 5.

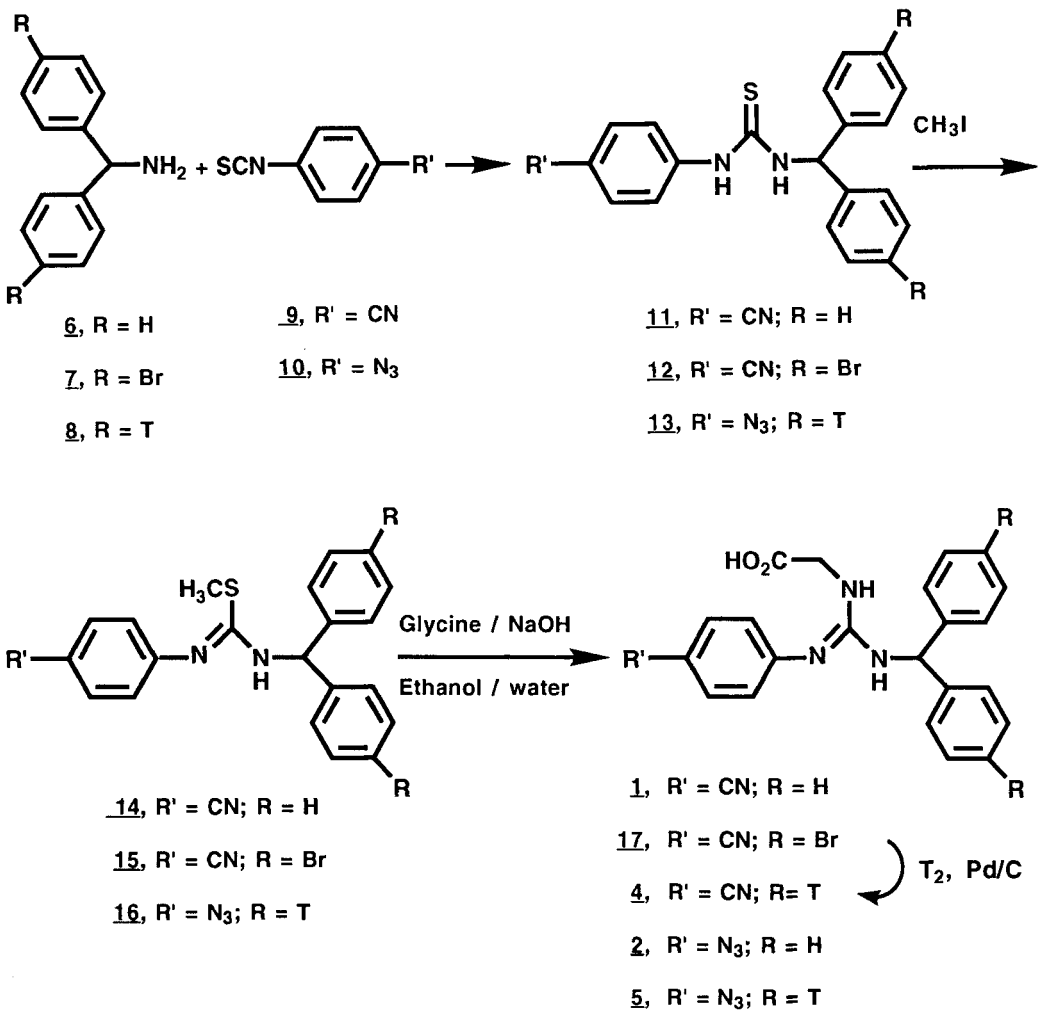
RESULTS AND DISCUSSION

Guanidineacetic acid 1 was synthesized on a 20 g scale (in overall yields above 80% for three steps) by reacting three equivalents of sodium salt of glycine with the isothiourea 14 in ethanol/water. Ditritioglycine (53.3 Ci/mmol) was used for the synthesis of radioactive guanidine 3 employing the same method but with one equivalent of labeled glycine. The overall yield of the product obtained was only 1.2%. During a second attempt to synthesize the compound 3, excess tritiated glycine was used in the reaction mixture to raise the yield. The desired product was obtained in significantly higher yield, but with considerable loss in radioactivity (>90% loss). We believe the loss of radioactivity is due to the use of excess glycine under the reaction conditions.

Catalytic hydrogenation of halo derivatives of guanidineacetic acid was then considered to circumvent this problem. This method has the advantage of introducing the label at any desired position by starting with the appropriately halogenated molecule. Depending on the number of halogen atoms in the molecule, very high specific activity could be incorporated in the substrate. The dibromoguanidineacetic acid 17 was chosen for tritiation and was synthesized as shown (from 7, via 12 and 15) in scheme 1. Catalytic hydrogenation (Pd/C) of the dibromoguanidine afforded the desired guanidine 1. The radiolabeled guanidine 4 was obtained using the same method but in the presence of tritium gas. The specific activity of the product obtained was determined to be 50.9 Ci/mmol.

For the synthesis of the tritiated photoaffinity labeling reagent, a different procedure had to be employed since catalytic hydrogenation (with tritium) is not compatible with the azido group. The synthesis of 5 was then designed with the radioactive starting material. Dibromobenzhydrylamine 7 was hydrogenolyzed in the presence of tritium gas and Palladium/Carbon catalyst to give the ditritio derivative 8. The ditritiated benzhydrylamine was then converted to the thiourea 13, and was carried on to the desired guanidine 5 via the isothiourea 16 as shown in scheme 1. The specific activity of the guanidine obtained was 48.8 Ci/mmol.

Scheme 1



The utilization of these labeled sweeteners for the study of sweet taste mechanism is currently in progress.

EXPERIMENTAL

General: Melting Points were obtained on a Thomas-Hoover Unimelt capillary apparatus and are not corrected. IR spectra were obtained on a Perkin Elmer

model 881 instrument. NMR spectra were obtained on a General Electric QE 300 instrument using tetramethylsilane as an internal standard. Mass spectrum (FAB) was obtained using a MAT 90 instrument. Microanalysis was performed by the Midwest Microlabs, Indianapolis, IN 46250. Chromatography refers to flash chromatography[5] on silica gel.

N-(p-Cyanophenyl)-N'-(diphenylmethyl)thiourea. (11). A mixture of p-cyanophenylisothiocyanate (8.74 g, 54.62 mmol) and aminodiphenylmethane (10.0 g, 54.57 mmol) in acetonitrile (150 mL) was heated at reflux for 4 h and the reaction mixture was concentrated. Upon addition of hexane-ethyl acetate (9:1) to the residue, a precipitate formed. The precipitate was filtered and recrystallized from hexane-ethyl acetate to afford 18.2 g (97%) of the desired product 11 as a white powder. mp 168-169 ^{o}C. ^{1}H NMR ($CDCl_3$) δ 6.81 (d, 1H, J=6 Hz), 7.25-7.36 (m, 10H), 7.54 (d, 2H, J=8 Hz), 7.87 (d, 2H, J=8 Hz). ^{13}C NMR ($CDCl_3$) δ 60.8, 105.9, 119.1, 121.3, 127.3, 127.6, 128.4, 132.4, 141.3, 143.9, 179.9.

Anal. Calcd. for $C_{21}H_{17}N_3S$: C, 73.44; H, 4.99; N, 12.24. Found: C, 73.13; H, 4.93; N, 12.24.

N-(p-Cyanophenyl)-N'-(diphenylmethyl)-S-methylisothiourea. (14). Iodomethane (20.0 g, 140.8 mmol) was added to a solution of the thiourea 11 (17.0 g, 49.56 mmol) in acetone (150 mL) and the reaction mixture was stirred at ambient temperature for 18 h. The reaction mixture was concentrated and the residue was partitioned between dichloromethane (500 mL) and sodium hydroxide (1N, 200 mL). The organic layer was separated, dried ($MgSO_4$) and concentrated to afford 16.0 g (90%) of the desired product as a crystalline solid. mp 141-142 ^{o}C. ^{1}H NMR ($CDCl_3$) δ 2.27 (s, 3H), 6.21 (m, 1H), 6.86 (d, 3H, J=8 Hz), 7.25-7.37 (m, 10H), 7.49 (d, 2H, J=7.3 Hz). ^{13}C NMR ($CDCl_3$) δ 14.3, 60.5, 105.3, 119.7, 122.7, 127.3, 127.6, 128.7, 133.0, 141.4.

Anal. Calcd. for $C_{22}H_{19}N_3S$: C, 73.92; H, 5.36; N, 11.76. Found: C, 73.51; H, 5.21; N, 11.64.

Synthesis of 3 with Tritium Labeled Glycine: Tritium labeled glycine (53.3 Ci/mmol, 5.0 mCi, received as a 0.01 N HCl solution) was lyophilized and the

residue was dissolved in 0.1 mL of 0.001 N NaOH (in water). This was added to a solution of 14 (68 μg, 0.190 μmoles) in ethanol and the reaction vessel was flame sealed. The sealed vial was heated at 80 °C for 18 h. No desired product was obtained at this point (HPLC). Further amount of 14 (200 μg, 0.559 μmoles) and NaOH (10 μL of 0.01 N NaOH) were added. The sealed vessel was heated at 80 °C for 18 h. The reaction mixture was purified by HPLC (Vydac C18, 4.6 X 250 mm, 30% acetonitrile (0.01% TFA) in water) and the peaks corresponding to the desired product were collected. The collected fractions were combined and the volume reduced in a stream of argon at room temperature. This removed most of the acetonitrile. The remaining solution was diluted with ethanol (2.3 mL) for a final volume of 5 mL. This provided 57 μCi of material at a radiochemical concentration of 11.4 μCi/mL. The radiochemical purity was determined as 89 ± 2%.

N-(p-Cyanophenyl)-N'-(4, 4'-dibromobenzhydryl)thiourea. (12). A mixture of the amine[4] 7, (3.7 g, 10.88 mmol) and p-cyanophenylisothiocyanate (9, 1.74 g, 10.88 mmol in acetonitrile was heated at reflux for 1 h, then concentrated. The residue was chromatographed (ethyl acetate : hexane 1:1) to afford the desired product 12 as a crystalline compound. Yield 5.2 g (96%). ^{1}H NMR ($CDCl_3$) δ 6.73 (d, 1H), 7.15 & 7.47 (AB, 8H, J=8.4 Hz)), 7.56 & 7.84 (AB, 4H, J=8.7 Hz). ^{13}C NMR ($CDCl_3$) δ 59.7, 106.1, 119.0, 121.4, 121.5, 129.2, 131.6, 132.5, 140.0, 143.8, 180.1.

N-(p-Cyanophenyl)-N'-(4, 4'-dibromobenzhydryl)-S-methylisothiourea. (15). Iodomethane (1.28 g, 9 mmol) was added to a solution of the thiourea 12 (1.5 g, 3 mmol) in acetone (25 mL) and the mixture was stirred for 24 h at room temperature. The reaction mixture was concentrated and the residue was dissolved in CH_2Cl_2 (200 mL) and 1N NaOH (100 mL). The organic layer was separated , dried and concentrated to afford 1.3 g (84%) of the desired product 15. ^{1}H NMR δ 2.23 (s, 3H), 6.21 (s, 1H), 7.16-7.47 (m, 12H).

N-(p-Cyanophenyl)-N'-(4, 4'-dibromobenzhydryl)guanidineacetic acid. (17). A solution of glycine (0.57 g, 7.5875 mmol) in NaOH (1N, 7.6 mL) was added to a solution of the isothiourea 15 (1.30 g, 2.529 mmol) in ethanol (25 mL) and the

reaction mixture was heated at reflux for 8 h, then concentrated. The residue was dissolved in NaOH (10 mL) and neutralized. The white precipitate formed was filtered, washed with water and dried to afford 1.2 g (88%) of the desired product 17. ^{1}H NMR (DMSO-d_6) δ 4.05 (s, 2H), 6.34 (s, 1H), 7.20 & 7.70 (AB, 4H, J=8.6 Hz), 7.26 & 7.55 (AB, 8H, J=8.4 Hz) . ^{13}C NMR δ 44.82, 58.6, 119.2, 121.2, 122.6, 130.0, 131.7, 133.7, 140.0, 154.2, 171.0.

Anal. Calcd for $C_{23}H_{18}N_4Br_2O_2.1.5H_2O$: C, 48.53; H, 3.72; N, 9.84; M= 540: Found: C, 48.41; H, 3.18; N, 9.84; m/e = 543 (M+2+H, FABMS).

N-(p-Cyanophenyl)-N'-(4, 4'-ditritiobenzhydryl)guanidineacetic acid. (4). The dibromo compound 17 (0.015 g) was dissolved in methanolic potassium hydroxide (750 μL) and was transferred to a tritiation vessel containing Pd/C (10%, 15 mg). The solution was stirred under an atmosphere of tritium gas (5 Ci) for 20 min. The catalyst was filtered off, washed with ethanol (10 mL) and the solution was rotary evaporated to dryness. Labile tritium was removed by co-evaporation with ethanol (3X10 mL). The residue was dissolved in ethanol giving a crude yield of 973 mCi at 70% radiochemical purity by thin layer chromatography. The product was purified by reverse phase HPLC. Calcd for $C_{23}H_{18}N_4O_2T_2$: M = 410. Found: m/e = 411 (M+H, FABMS).

4, 4'-Ditritiobenzhydrylamine. (8). A mixture of dibromobenzhydrylamine 7 (51.8 mg, 0.152 mmol) and Pd/C (14.1 mg, 10%) and Pd/$CaCO_3$ (25.4 mg) in methanol (2 mL) in a hydrogenation flask (5 mL) was connected to a tritiation manifold and degassed. Tritium gas (20 Ci) was introduced to the flask and the reaction mixture was stirred rapidly at 20 °C for 20 min. The reaction mixture was filtered and the filtrate was washed with methanol:THF (20 mL, 1:1). The filtrate was concentrated to dryness in vacuo at 20 °C. The labile tritium was removed by successive rotary evaporation with ethanol to yield 5.63 Ci of the desired material. The material was purified by HPLC [Macro Dynamax ODS column; Gradient of 100% solvent A (methanol:water: triethylamine 50:50:1) to 100% solvent B (methanol:water: triethylamine 80:20:1) over a period of 60 minutes; flow rate 9 mL/min] to afford 2.07 Ci of the ditritiobenzhydrylamine.

N-(p-Azidophenyl)-N'-(4, 4'-ditritiobenzhydryl)thiourea. (13). A mixture of p-azidophenylisothiocyanate (0.050 g, 0.284 mmol) and [^{3}H] benzhydrylamine (8, 2.07 Ci, 50 Ci/mmol, 0.041 mmol) in acetonitrile (5 mL) was stirred under nitrogen at room temperature for 2 h. The reaction mixture was concentrated and was carried to the next step without further purification.

N-(p-Azidophenyl)-N'-(4, 4'-ditritiobenzhydryl)-S-methylisothiourea. (15). Iodomethane (100 µL, 1.61 mmol) was added to a acetone solution of the thiourea 13 (2.07 Ci) and was stirred for 18 h at room temperature. The reaction mixture was concentrated to dryness and the residue was dissolved in dichloromethane (50 mL). The organic phase was extracted with sodium hydroxide (20 mL, 1M), saturated sodium thiosulfate (20 mL), water (3X20 mL) and dried ($MgSO_4$). The organic layer was concentrated to afford 1.49 Ci of the desired isothiourea. The product was carried to the next step without further purification.

N-(p-Azidophenyl)-N'-(4, 4'-ditritiobenzhydryl)guanidineacetic acid. (5). A solution of glycine (0.00675 g, 0.09 mmol) in water (1.0 mL) and sodium hydroxide (0.0036 g, 0.09 mmol in 0.5 mL water) was added to a solution of the isothiourea (1.0 Ci, 0.02 mmol, ~50 Ci/mmol) in ethanol (5 mL). The reaction mixture was heated at reflux for 5 h in an oil bath at 120 °C. Glacial acetic acid (10 µL) was added to the cooled reaction mixture and diluted to 50 mL with ethanol: water (5:1) and stored at -20 °C. The reaction mixture was concentrated to dryness and the residue was dissolved in dichloromethane (50 mL). The organic layer was washed with water (100 mL). The aqueous layer was extracted with dichloromethane (4X25 mL) and the combined organic layer was dried ($MgSO_4$) and concentrated to afford 554 mCi of the product. The residue was dissolved in ethanol (0.5 mL) and purified by preparative TLC (methanol: water: acetic acid 80:20:1). The desired material was extracted with ethanol (50 mL). The ethanol solution was filtered and concentrated to afford 176 mCi of the product. Further purification was accomplished using HPLC (Ultrasphere ODS column, methanol: water: triethylamine 50:50:0.1, 3 mL/min) to afford 79 mCi of the desired product. The

radiochemical purity was established to be >98%. The specific activity of the product was 48.8 Ci/mmol.

Anal. Calcd for $C_{22}H_{18}T_2N_6O_2$: M= 404. Found: m/e = 405 (M+1, FABMS)

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REFERENCES

1. (a). Sato, M. - Jpn. J. Physiol. 35: 875 (1985) and references cited therein. (b). Shimazaki, K.; Sato, M.; Nakao, M. - Biochim. Biophys. Acta. 884: 291 (1986). (c). Cagan, R. H.; Morris, R. W. - Proc. Natl. Acad. Sci. USA. 76: 1692 (1979).

2. J-M Tinti and C. Nofre, p 88. - In Sweeteners. Discovery, Molecular Design, and Chemoreception. Ed. D. E. Walters, F. T. Orthoefer, G. E. DuBois. ACS Symposium Series 450. American Chemical Society, Washington, DC 1991 and references cited therein.

3. S. Nagarajan, M. S. Kellogg, G. E. DuBois, G. Hellekant. - J. Med. Chem. (In preparation)

4. G. R. Clemo and G. A. Swan. - J. Chem. Soc: 370 (1942).

5. Still, W. C.; Kahn, M.; Mitra, A. - J. Org. Chem. 43: 2923 (1978).