Understanding the Mechanism of Sweet Taste: Synthesis of Ultrapotent Guanidinoacetic Acid Photoaffinity Labeling Reagents[1]

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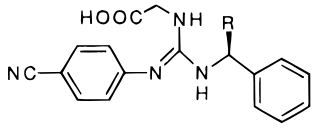
Azido-functionalized analogs of potently sweet guanidinoacetic acids have been synthesized for use as sweetener receptor photoaffinity labeling reagents. These compounds have been synthesized using readily available starting materials. One of the azido-labeled guanidinoacetic acids has been evaluated in an electrophysiological model in the Rhesus monkey. We found that the photoaffinity-labeling reagent caused irreversible inhibition in electrophysiological response to sweeteners upon exposure of the monkey tongue to a combination of the reagent and UV light.

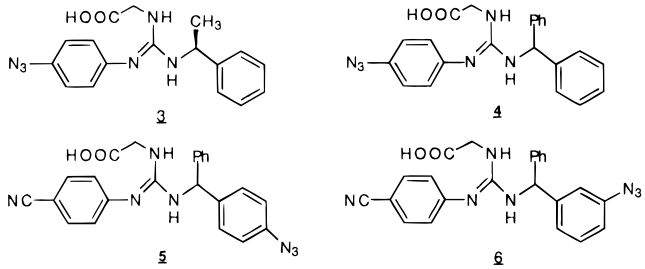
The perception of sweet taste is assumed to be initiated by the binding of the sweet stimuli to receptor molecules on the surface of the tongue. Results of many experiments strongly argue for sweet taste mediation by multiple molecular recognition units. These multiple molecular recognition units may be different receptors, although other rationales are also possible. Attempts have been made in the past to isolate receptors from bovine, rodent, and even primate sources.[2] These efforts have been difficult due to the lack of potent agonists or antagonists. The identification by Nofre and Tinti[3] of the guanidinoacetic acid class of sweeteners, with some representatives exhibiting potencies in excess of 100 000 times that of sucrose, suggested to us that preparation of the necessary molecular probes may now be feasible. We decided to investigate the guanidinoacetic acid sweetener receptor by employing photoaffinity-labeling techniques.[4] We disclose here the synthesis of four photoaffinity-labeling reagents and preliminary results from labeling experiments.

Our photoaffinity-labeling reagents are based on the templates of guanidinoacetic acids **1** and **2**. The selection of **1** is based on the finding that it is potently active in both primates and rodents[5] and the selection of **2** based on the very high potency in humans.[1] These guanidinoacetic acids **1** and **2** have been estimated to be 28 000[3] (vs 2% standard solution of sucrose) and 200 000[§] (vs 2% sucrose) times as potent as sucrose, respectively.[6]



1, R = CH3
2, R = Ph

A useful photoaffinity probe must exhibit a high level of activity. Thus the position of attachment of the photosensitive functionality to the structural core must not significantly affect sweetness potency. On the basis of the known structure−activity relationships (SAR) of the guanidinoacetic acids, we felt that sweetness potency would be maintained by substitution of a photolabile azide moiety for the nitrile groups of **1** and **2**. This speculation is based on the correlation between sweetness potencies and the Hammet σ constants of groups substituted in this position and the σ constant (+0.42) of the azido group.[7] Thus, we elected to synthesize **3** and **4**. Synthesis of reagents with photolabel substitu-



3 **4** **5** **6**

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§ The determination of sweetness potencies is most accurately accomplished by paired-difference testing of a narrow range of sweetener concentrations relative to the sucrose reference. Such testing must be done with a large panel (e.g., 40−50 subjects), the members of which have demonstrated proficiency in sweetness intensity scaling. However, for experimental compounds on which limited safety assessment work has been completed, we employ an estimation procedure based on small panels (e.g., three or four subjects). Subjects are trained to recognize the sweetness intensities of sucrose reference in the range of 2−10%. Subjects then rate the sweetness intensities of several dilutions of the sweetener where the dilutions are chosen to span the sweetness intensities of the sucrose reference of interest. For the case of **2**, a concentration of 0.10 ppm was judged to be approximately equivalent to a 2.0% sucrose reference [Pw(2)] and is calculated to be 200000× sucrose. There are two special considerations which complicated the sweetness potency of **2**. (1) It is well-known that guanidinoacetic acid compounds often exist in equilibrium with acyl guanidine cyclic dehydration products (e.g., creatine/creatinine equilibrium; see: *Merck Index*, 10th ed.; Windholz, M., Ed.; Merck & Co.: Rahway, NJ, 1983; p 2551). For this reason, **2** was dissolved under very mild conditions: Specifically, a 25 mg/25 mL solution was prepared in 1/1 EtOH/H_2O with minimal warming. Aqueous dilutions were made as soon as complete dissolution was achieved and they were then tasted immediately. (2) The guanidinoacetic acid **2** exhibits a sweetness which is notably slow in onset relative to sucrose, particularly when evaluated at low levels of sweetness intensity. For **2** at 0.10 ppm, no sweetness is noted for the first ∼10−15 s, after which a gradual rise in sweetness is observed, maximizing at ∼30−45 s. Thus it is difficult for tasters to decide when the sweetness maxima has been reached, and in fact, unless the solution is held in the mouth for the requisite time, tasters report the observation of no sweetness at all.

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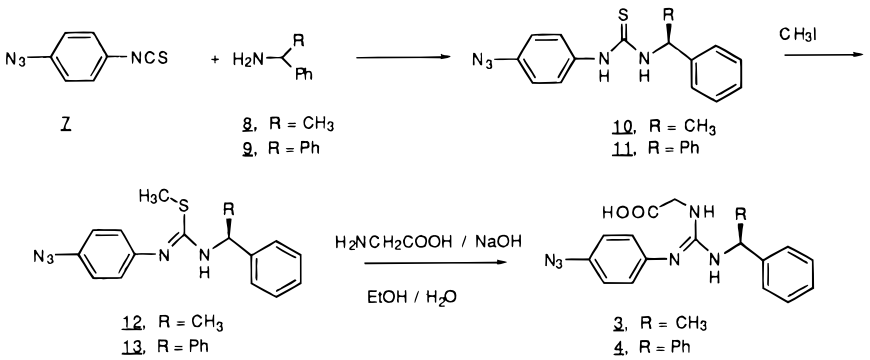
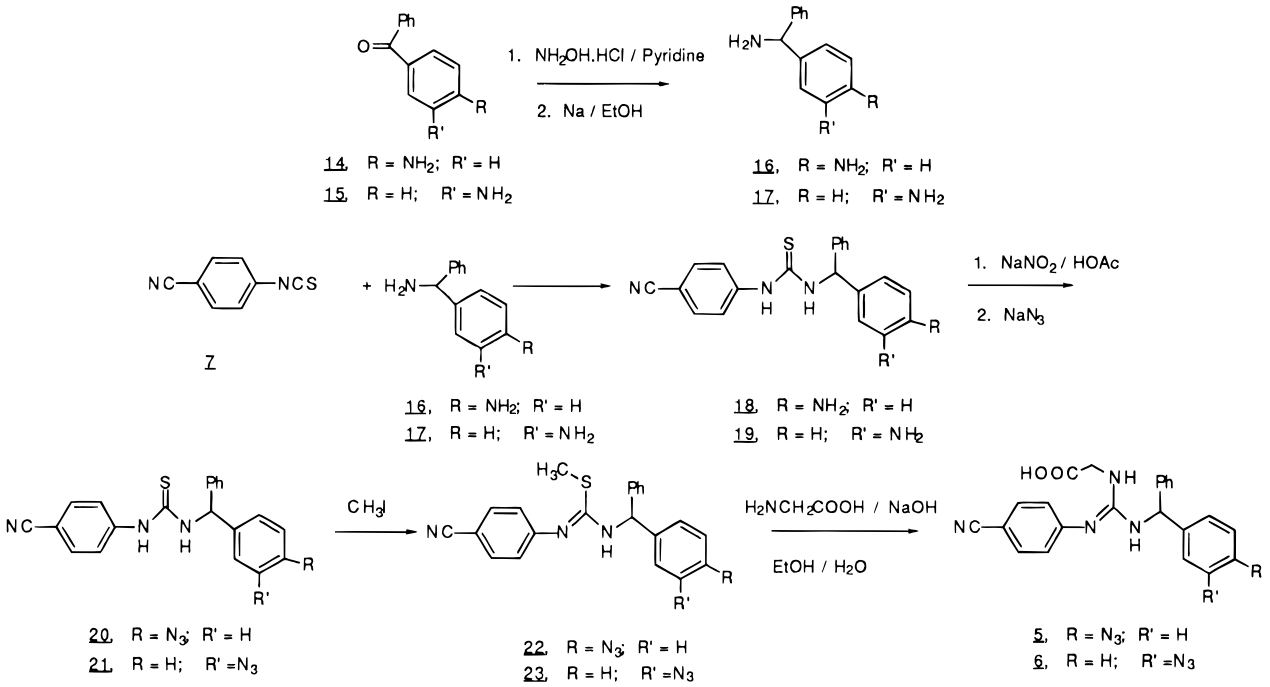
Scheme 1



Scheme 2



tion at the ortho or meta positions of the anilines was not pursued since the ortho analogs are not sweet[3] and the precursors for the meta analogs are not readily available. Substitution on the acetic acid moiety reduces sweetness potency drastically and was also not considered.[3] From inspection of the SAR of guanidinoacetic acid sweeteners, it is also clear that the substitution at the hydrophobic moiety may be varied a great deal without significantly affecting sweetness activity.[3] Thus, in addition to azide- substituted guanidinoacetic acids **3** and **4** we also elected to synthesize **5** and **6** with the expectation that they also would be potently sweet.

Photolabeling reagent **3** was synthesized as shown in Scheme 1 by starting with commercially available *p*-azidophenyl isothiocyanate (**7**). Reaction of **7** with (*S*)-α-phenethylamine (**8**) gave thiourea **10**. The thiourea **10** was converted to isothiourea **12** by treatment with methyl iodide. Reaction of isothiourea with sodium salt of glycine afforded the desired guanidinoacetic acid **3** in 68% overall yield. The synthesis of **4** was accomplished in 42% overall yield as illustrated in Scheme 1, employing a similar route starting from benzhydrylamine.

The reagents **5** and **6** in which the azido group is located in the 4- and 3- positions, respectively, of the benzhydryl portion of guanidine **2** were synthesized (Scheme 2), employing a procedure similar to that described above. The corresponding 4- and 3-aminobenzhydrylamines (**16**) and (**17**) were synthesized from commercially available 4-aminobenzophenone and 3-aminobenzophenone, respectively, via reduction of their oximes. Reaction of these amines with *p*-cyanophenyl isothiocyanate provided the amino-substituted thioureas **18** and **19**. These aromatic amines were transformed to the azides **20** and **21** by diazotization, followed by reaction with sodium azide. From the azidothioureas, the guanidinoacetic acids **5** and **6** were then synthesized via corresponding isothioureas **22** and **23** as was accomplished earlier for **3** and **4** (Scheme 2). The taste activities of aryl azides **3**−**6** show that our initial assumptions were indeed correct. We found that all four compounds synthesized are sweet. The sweetness potencies of **3**−**6** were estimated, in humans, to be as follows: **3**, 1000 (1%); **4**, 30 000 (3%); **5**, 30 000 (3%); and **6**, 30 000 (3%) times that of sucrose. The sweetness potencies were determined by a trained human taste panel, and the percentages in parentheses are the

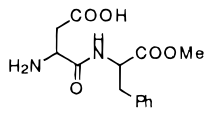
sucrose reference concentrations judged to be equisweet with the X-fold (X = potency) more dilute guanidinoacetic acid sweeteners.

The *in vitro* photolysis of guanidinoacetic acid **4** was examined to demonstrate that the reagent is photolabile and to determine the minimum time required for photolysis. The *in vitro* photolysis of **4** was conducted with a 450 W medium-pressure mercury vapor lamp in ethanol in the presence of dimethylamine in the hope of trapping the nitrene intermediate.[8] The consumption of **4** was complete in less than 2 min. NMR and mass spectral analysis of the photolysis mixture demonstrated the presence of a nitrene−dimethylamine adduct.

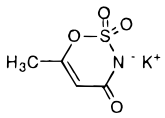
Receptor-labeling studies in animals to date have been primarily limited to **4**. The details of these experiments will be described elsewhere. Monkeys were used in these experiments since the sense of taste in nonprimates differs markedly from that of humans.[9a] The sweet compounds synthesized and used in this study are sweet to old world monkeys and to humans. The taste nerve impulses have been recorded from the chorda tympani proper (CT) nerve, which mediates much of the sweet taste response following application of the sweeteners on the tongue. The nerve impulses can be measured by employing electrophysiological methods. The protocols for surgically exposing the chorda tympani nerve in anesthetized animals and measuring and recording nerve responses have been developed in our laboratories (Wisconsin; see Methods).[9]

We then attempted to photolable the guanidinoacetic acid receptor on the tongue of live anesthetized monkeys. The experimental protocol we employed to assess the effects of *in vivo* photolabeling with **4** on the electrophysiological responses to sweeteners constituted the following steps: (1) A range of sweet-tasting stimuli were applied to the tongue of the animal and the summated nerve rsponses determined; (2) guanidinoacetic acid **4** was placed on the tongue, after which the tongue was irradiated for 40 s [employing a hand held pencil UV light (254 nm) with provisions for limiting exposure to other parts of the animal and the user]. The UV lamp was held at 1 cm above the tongue over the area to be irradiated; (3) step 2 was repeated two times; (4) the tongue was washed, and the summated CT response to the sweet-tasting stimuli were again determined.

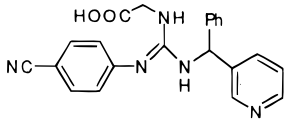
Concentration−response functions were determined for the taste stimulants aspartame (APM) and acesulfame-K (ACE-K) by using solutions these sweeteners of varying concentrations. The concentration-



Aspartame



Acesulfame-K



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response functions were determined for aspartame (Figure 1) and acesulfame-K (Figure 2) before and after photoaffinity labeling. A decrease in summated nerve response over a range of concentrations for both sweeteners was observed after the photolabeling. An experiment was designed to saturate all the putative sweet receptors in the tongue by exposing it to high concentrations of **24** [sweetness potency = 30 000 (compared to 3% sucrose), synthesized as shown in Scheme 1 by starting from (*R*,*S*)-α-(3-pyridyl)benzylamine]. The compound **24** was chosen because of its relatively high water solubility compared to other sweeteners in this class. When irradiation of the tongue was attempted with **4** (10 mg/L) in the presence of excess **24** (100 mg/L), no labeling of the tongue was observed as indicated by the unchanged nerve response for ACE-K. However, when the tongue was exposed to the combination of **4** (10 mg/L) and UV light, in the same experiment, a significant drop in response was observed for ACE-K (Figure 2). We have shown from this experiment that **24** which is structurally similar to **4** competes effectively with **4** and prevents the photolabeling of the tongue.

We performed several control experiments. Exposing the tongue to UV light alone did not cause any decrease of response to taste stimulants (to show that exposure

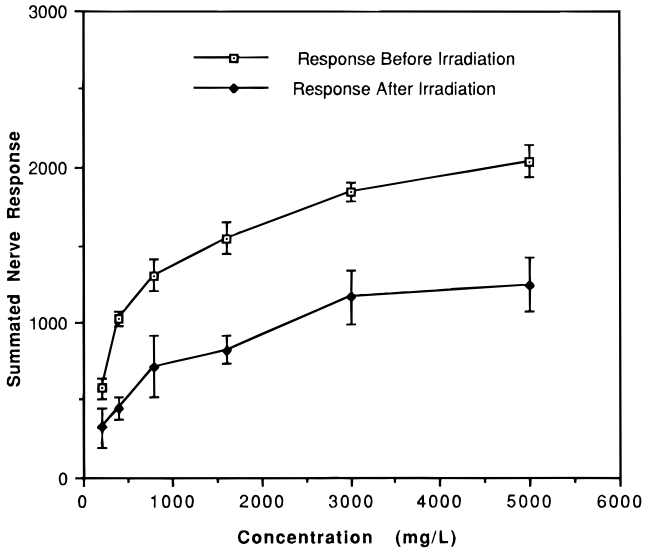

Figure 1. Nerve response in monkey for aspartame before and after irradiation of the tongue with UV light and **4**.

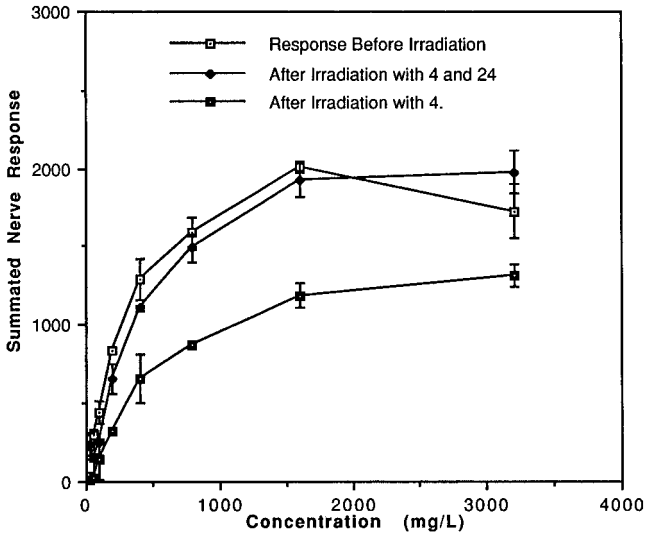

Figure 2. Nerve response in monkey for ACE-K before irradiation of the tongue, after irradiation with a mixture of **4** and **24**, and followed by irradiation with **4** alone.

of tongue to UV (3 × 40 s) light does not cause damage). Repeated application of the photoaffinity-labeling reagent, or the application of preirradiated reagent, did not cause any decrease in response to taste stimulants (to show that either the reagent itself or the product of irradiation does not cause irreversible inhibition of taste response). Application of a potently sweet non-photosensitive guanidine (**2**) followed by exposure to UV light (to show that the guanidineacetic acid class of sweeteners do not provide active intermediates upon exposure to UV light) did not reduce the response in monkeys for the various stimulants. These control experiments clearly demonstrated that only the combination of the photolabel and the UV light affected the ability of these monkeys to respond to various stimulants.

In conclusion, we have found that the photoaffinity-labeling reagent caused irreversible inhibition in response for the sweeteners aspartame and acesulfame-K. Further work is needed to show that the photolabeling is taking place at the putative sweet receptor. Tritium-labeled **4** has recently been synthesized in our laboratories,[10] and the labeling experiments in tongue preparations to isolate the photolabeled macromolecules are in progress.

Experimental Section

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 in silica gel. A hand held pencil UV light (Spectronics Model 11SC-1, 254 nm) with provisions for limiting exposure to other parts of the animal and the user was employed for the animal experiments.

Methods. The technique to expose the CT nerve will be briefly desribed here: Monkeys (M. mulatta) were anesthetized with an im injection of ketamine (400 mg/animal) and atropine (0.5 mg/animal). They were then intubated and maintained on a mixture of oxygen and halothane. Fluid was replenished with iv 5% dextrose and lactated Ringers solution. The right CT nerve was exposed through an incision along the mandibular angle between the rostal lobes of the partoid gland and the mandibular bone. First, the tissue attached to the mandibular angle was sectioned. Then blunt dissection was used to follow the caudiomedial side of the pterygoid muscle down to its origin at the pterygoid plate of the skull and to the CT nerve. After the experiment, the wound was closed with chrome nylon. The animals recovered, and the nylon sutures were removed after 7−10 days.

The nerve impulse activity was recorded with a PAR 113 amplifier, monitored over a loud speaker and an oscilloscope, and fed into a recorder (Gould ES 1000) and an IBM PC-AT computer via a DAS-Keithly interface. The activity in the whole nerve preparation was displayed as a summated signal through an absolute value integrator. The summated signals and a code related to each tastant on the tongue was fed in to the computer. The computer program sampled the summated response before, during, and after stimulation and displayed it on an oscilloscope. The stimulation time and the sequence of the tastants can be modified and controlled by the computer. The stimulus name, its order, the maximum amplitude and surface area of each response, the level of nerve activity before each stimulation, and the time for each stimulation was continually printed out during the experiment. An early version of the stimulation equipment and recording technique used has been described. The parameters measured on the summated nerve trace has also been defined and discussed in detail elsewhere.[9] We have made use of this technique to perform our photoaffinty-labeling experiments in Rhesus monkeys.

***N*-(4-Azidophenyl)-*N*′-(diphenylmethyl)thiourea (11).** A mixture of *p*-azidophenyl isothiocyanate (**7**, 0.50 g, 2.84 mmol) and benzhydrylamine (**9**, 0.52 g, 2.84 mmol) in acetonitrile (10 mL) was stirred at room temperature for 2 h, and the precipitate was filtered. The solid was recrystallized from hexane−ethyl acetate to afford 0.92 g (90%) of the desired product as pale orange powder: mp 175 °C dec; ^{1}H NMR ($CDCl_3$) δ 6.97 (s, 1H), 7.01 (d, 2H, J = 8.3 Hz), 7.17−7.37 (m, 12H); ^{13}C NMR ($CDCl_3$) δ 61.5, 119.6, 125.9, 127.2, 128.3, 133.6, 137.7, 140.6, 180.1; IR (Nujol) 2126 cm^{-1}. Anal. ($C_{20}H_{17}N_5S$) C, H, N.

***N*-(4-Azidophenyl)-*N*′-[(S)-α-phenethyl]thiourea (10).** This was prepared using the above procedure with *p*-azidophenyl isothiocyanate and (*S*)-α-phenethylamine: yield 95%; mp 117 °C; ^{1}H NMR ($CDCl_3$) δ 1.53 (d, 3H, J = 6.9 Hz), 4.79 (br s, 1H), 7.0 and 7.17 (AB quartet, 4H, J = 8.7 Hz), 7.28−7.36 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 21.5, 54.1, 120.2, 126.0, 126.5, 126.6, 127.5, 128.7, 138.6, 141.9, 179.4. Anal. ($C_{15}H_{15}N_5S$) C, H, N.

***N*-(4-Azidophenyl)-*N*′-(diphenylmethyl)-*S*-methylisothiourea (13).** Iodomethane (1.01 g, 7.11 mmol) was added to a solution of the thiourea (**11**, 0.85 g, 2.37 mmol) in acetone (15 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 (50 mL) and sodium hydroxide (1 N, 20 mL). The organic layer was separated, dried ($MgSO_4$), and concentrated to afford 0.88 g (99%) of the desired product as an oil: ^{1}H NMR ($CDCl_3$) δ 2.15 (s, 3H), 6.22 (s, 1H), 6.80, 6.90 (AB quartet, 4H, J = 8.3 Hz), 7.18−7.36 (m, 10H); IR (Nujol) 2118 cm^{-1}. Anal. ($C_{21}H_{19}N_5S$) C, H, N.

***N*-(4-Azidophenyl)-*N*′-[(*S*)-α-phenethyl]-*S*-methylisothiourea (12).** Using the above procedure thiourea **10** was converted to isothiourea **12**: yield 88%; ^{1}H NMR ($CDCl_3$) δ 1.51 (d, 3H, J = 6.5 Hz), 2.24 (s, 3H), 4.63 (m, 1H), 6.84 and 6.93 (AB, 4H, J = 8.7 Hz), 7.23−7.37 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 14.2, 22.7, 52.2, 119.5, 119.6, 123.4, 126.0, 127.3, 128.7, 134.1, 143.7, 146.8. Anal. ($C_{16}H_{17}N_5S$) C, H, N.

***N*-(4-Azidophenyl)-*N*′-(diphenylmethyl)guanidinoacetic Acid (4).** A solution of glycine (0.531 g, 7.078 mmol) and NaOH (0.283 g, 7.078 mmol) in water (5 mL) was added to a solution of the isothiourea (**13**, 0.88 g, 2.359 mmol) in ethanol (25 mL), and the mixture was heated at reflux for 6 h and then concentrated. The residue was dissolved in water (50 mL), and the solution was washed with CH_2Cl_2 (20 mL). The pH of the aqueous layer was adjusted to 6. The precipitate formed was collected, dried, and recrystallized from ethanol−water to afford 0.440 g (47%) of the desired product as a pale yellow powder: mp 167−168 °C dec; ^{1}H NMR (DMSO-d_6) δ 3.53 (s, 2H), 5.96 (s, 1H), 7.03 (s, 4H), 7.31 (s, 10H); ^{13}C NMR (DMSO-d_6) δ 47.6, 59.5, 119.7, 120.2, 125.2, 127.3, 127.5, 127.8, 128.9, 129.0, 141.7, 153.9, 172.1; IR (Nujol) 2120 cm^{-1}. Anal. ($C_{22}H_{20}N_6O_2 \cdot 0.75H_2O$) C, H, N.

***N*-(4-Azidophenyl)-*N*′-[(*S*)-α-phenethyl]guanidinoacetic acid (3).** Using the above procedure, isothiourea **12** was converted to **13**: yield 81%; mp 184−186 °C dec; ^{1}H NMR (DMSO-d_6) δ 1.43 (d, 3H), 3.52 (s, 2H), 4.82 (m, 1H), 7.03 and 7.12 (AB, 4H, J = 8.7 Hz), 7.22−7.33 (m, 5H); IR (Nujol) 2120 cm^{-1}. Anal. ($C_{17}H_{18}N_6O_2$) C, H, N.

***p*-Aminobenzhydrylamine (16). Synthesis of *p*-Aminobenzhydryl Ketoxime.** A mixture of *p*-aminobenzophenone (50.0 g, 253.8 mmol) and hydroxylamine hydrochloride (35.25 g, 506 mmol) in pyridine (600 mL) was heated at reflux for 48 h. The pyridine was removed in vacuo, and the residue was partitioned between water (400 mL) and dichloromethane (1 L). The organic layer was dried ($MgSO_4$) and concentrated to afford 52 g (97%) of the desired oxime as a crystalline solid.

Reduction of the Ketoxime. Shiny pieces of sodium (36.0 g, 1.5 mol) were added to a hot solution of the oxime (52.0 g, 0.245 mmol) in dry ethanol (600 mL). After all the sodium had dissolved, the reaction mixture was cooled, diluted with water (1 L), and extracted with ether (3 × 600 mL). The combined organic layers were dried ($MgSO_4$) and concentrated

to afford 44.0 g (91%) of the desired amine as an oil. The diamino compound was carried to the next stage without further purification.

***m*-Aminobenzhydrylamine (17)** was synthesized using a similar procedure starting with *m*-aminobenzophenone: yield 84%; ^{1}H NMR ($CDCl_3$) δ 5.06 (s, 1H), 6.49−7.39 (m, 9H). The diamino compound was carried to the next stage without further purification.

***N*-(4′-Azidobenzhydryl)-*N*′-(4-cyanophenyl)thiourea (20): Synthesis of *N*-(4′-Aminobenzhydryl)-*N*′-(4-cyanophenyl)thiourea (18).** A mixture of *p*-cyanophenyl isothiocyanate (35.55 g, 222.2 mmol) and *p*-aminobenzhydrylamine (44.0 g, 222.2 mmol) in acetonitrile (900 mL) was heated at reflux for 2 h, and the reaction mixture was concentrated. The residue was purified by chromatography (ethyl acetate−hexane, 1:1) to afford the desired thiourea as a pale yellow powder.

Conversion of *N*-(4′-Aminobenzhydryl)-*N*′-(4-cyanophenyl)thiourea (18) to *N*-(4′-Azidobenzhydryl)-*N*′-(4-cyanophenyl)thiourea (20). A solution of sodium nitrite (2.10 g, 30.60 mmol) in water (50 mL) was added to a cooled (0−5 °C) suspension of the thiourea (10.0 g, 27.90 mmol) in glacial acetic acid (100 mL). The color of the reaction mixture changed to deep yellow. After 15 min at this temperature, a solution of sodium azide (10.0 g, 153.85 mmol) in water (50 mL) was added over a period of 10 min. The color of the reaction mixture changed slowly to off-white, and the stirring was continued for an additional 1 h. Water (200 mL) was added to the reaction mixture, and the precipitate was filtered, washed with water, and dried. Chromatography of the solid (ethyl acetate−hexane, 3:7) afforded 5.2 g (49%) of the desired azido compound as a gum which slowly solidified: mp 75−76 °C; ^{1}H NMR ($CDCl_3$) δ 6.95 (d, 2H, J = 8.5 Hz), 7.08 (d, 1H, J = 7.6 Hz), 7.18−7.33 (m, 7H), 7.40 (d, 2H, J = 8.7 Hz), 7.50 (d, 2H, J = 8.7 Hz); ^{13}C NMR ($CDCl_3$) δ 64.6, 79.5, 80.0, 110.9, 121.5, 122.3, 125.8, 130.3, 131.0, 131.8, 131.9, 136.2, 144.8, 182.5; IR (Nujol) 2122, 2227 cm^{-1}. Anal. ($C_{21}H_{16}N_6S$) C, H, N.

***N*-(3′-Azidobenzhydryl)-*N*′-(4-cyanophenyl)thiourea (21): Synthesis of *N*-(3′-Aminobenzhydryl)-*N*′-(4-cyanophenyl)thiourea (19).** This was synthesized starting with *m*-aminobenzhydrylamine and employing the procedure described above: mp 90 °C; ^{1}H NMR ($CDCl_3$) δ 6.7 (m, 1H), 6.87 (s, 1H), 6.93−7.02 (m, 2H), 7.20−7.35 (m, 6H), 7.48 (AB, 4H, J = 1.8 Hz); ^{13} C NMR ($CDCl_3$) δ 62.0, 108.2, 118.2, 118.5, 118.8, 123.0, 124.1, 127.7, 128.4, 129.2, 130.4, 133.6, 140.8, 142.0, 179.7; IR (Nujol) 2120, 2223 cm^{-1}. Anal. ($C_{21}H_{16}N_6S$) C, H, N.

***N*-(4′-Azidobenzhydryl)-*N*′-(4-cyanophenyl)-*S*-methylisothiourea (22).** Iodomethane (4.4 g, 30.1 mmol) was added to a solution of the thiourea (**20**, 4.0 g, 10.42 mmol) in acetone (100 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 (100 mL) and sodium hydroxide (1 N, 20 mL). The organic layer was separated, dried ($MgSO_4$), and concentrated to afford 3.40 g (82%) of the desired product as an oil which solidified upon cooling: mp 109−110 °C; ^{1}H NMR ($CDCl_3$) δ 2.26 (s, 3H), 6.18 (s, 1H), 6.86 & 7.35 (AB, 4H, J = 8.1 Hz), 7.01, 7.50 (AB, 4H, J = 8.5 Hz), 7.23−7.47 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 14.4, 59.9, 105.5, 119.3, 119.7, 122.7, 127.4, 127.8, 128.9, 133.1, 138.2, 139.3. Anal. ($C_{22}H_{18}N_6S$) C, H, N.

***N*-(3′-Azidobenzhydryl)-*N*′-(4-cyanophenyl)-*S*-methylisothiourea (23).** With thiourea **21** as the starting material, using the procedure above, **23** was synthesized: yield 71%; mp 135 °C; ^{1}H NMR ($CDCl_3$) δ 2.27 (s, 3H), 6.18 (s, 1H), 6.87 (d, 2H, J = 8.2 Hz), 6.93−7.06 (m, 3H), 7.23−7.36 (m, 6H), 7.59 (d, 2H, J = 2 Hz); ^{13}C NMR ($CDCl_3$) δ 17.2, 63.1, 108.3, 120.7, 120.9, 122.4, 125.5, 126.6, 130.2, 130.8, 131.7, 132.9, 135.9, 143.3. Anal. ($C_{22}H_{18}N_6S$) C, H, N.

***N*-(4′-Azidobenzhydryl)-*N*′-(4-cyanophenyl)guanidinoacetic Acid (5).** A solution of glycine (3.0 g, 23.44 mmol) and NaOH (0.94 g, 23.44 mmol) in water (10 mL) was added to a solution of the isothiourea (**22**, 3.0 g, 7.812 mmol) in ethanol (100 mL), and the reaction mixture was heated at reflux for 6 h and then concentrated. The residue was dissolved in water (50 mL), and solution was washed with CH_2Cl_2 (20 mL). The pH of the aqueous layer was adjusted to 6. The precipitate was collected, dried, and recrystallized from ethanol−water to afford 3.0 g (90%) of the desired product as pale yellow powder: mp 150−152 °C dec; ^{1}H NMR (DMSO-d_6) δ 3.65 (s, 2H), 5.99 (s, 1H), 6.96 (d, 2H, J = 8.5 Hz), 7.13 (d, 2H, J = 8.5 Hz), 7.21−7.30 (m, 7H), 7.56 (d, 2H, J = 8.6 Hz); ^{13}C NMR (DMSO-d_6) δ 47.5, 59.7, 106.0, 119.4, 119.6, 122.2, 127.5, 128.2, 128.3, 129.1, 129.3, 133.8, 139.2, 140.6, 144.1, 154.5, 172.2; IR (Nujol) 2120, 2226 cm^{-1}. Anal. ($C_{23}H_{19}N_7O_2 \cdot 0.25H_2O$) C, H, N.

***N*-(3′-Azidobenzhydryl)-*N*′-(4-cyanophenyl)guanidinoacetic Acid (6).** With isothiourea **23** as the starting material, the guanidineacetic acid **6** was synthesized in 89% yield: mp 144−145 °C dec; ^{1}H NMR (DMSO-d_6) δ 3.58 (s, 2H), 6.00 (s, 1H), 6.93 (d, 1H, J = 7.9 Hz), 6.99 (s, 1H), 7.04 and 7.52 (AB, 4H, J = 8.4 Hz), 7.11 (d, 1H, J = 7.8 Hz), 7.2−7.32 (m, 5H); ^{13}C NMR (DMSO-d_6) δ 47.5, 59.7, 104.8, 118.2, 118.5, 119.7, 122.4, 124.4, 127.6, 128.0, 128.1, 128.9, 129.1, 130.7, 133.7, 140.1, 141.0, 143.7, 154.3, 172.4; IR (Nujol) 2120, 2220 cm^{-1}. Anal. ($C_{23}H_{19}N_7O_2 \cdot H_2O$) C, H, N.

***N*-(*p*-Cyanophenyl)-*N*′-[(*R*,*S*)-α-(3-pyridyl)benzyl]thiourea (25).** A mixture of *p*-cyanophenyl isothiocyanate (18.8 g, 117.5 mmol) and (*R*,*S*)-α-(3-pyridyl)benzylamine[11] (21.6 g, 117.4 mmol) in acetonitrile (400 mL) was stirred at ambient temperature for 6 h to give a white precipitate. The precipitate was filtered, washed with ether, and dried to afford 37.9 g (94%) of the desired product as a white powder: mp 169 °C; ^{1}H NMR ($CDCl_3$) δ 6.84 (s, 1H), 7.25−7.38 (m, 6H), 7.53 and 7.85 (AB, 4H, J = 8.5 Hz), 7.63 (m, 1H), 8.47 (dd, 1H, J = 1.5 and 4.8 Hz), 8.56 (d, 1H, J = 2.1 Hz); ^{13}C NMR ($CDCl_3$) δ 58.6, 105.8, 118.4, 121.1, 123.2, 127.5, 128.5, 132.2, 135.1, 136.9, 140.0, 143.4, 147.9, 148.4, 179.7. Anal. ($C_{20}H_{16}N_4S$) C, H, N.

***N*-(*p*-Cyanophenyl)-*S*-methyl-*N*′-[(*R*,*S*)-α-(3-pyridyl)benzyl]isothiourea (26).** Iodomethane (5.0 g, 35.21 mmol) was added to a solution of the thiourea **25** (4.0 g, 11.63 mmol) in acetone (100 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 (100 mL) and sodium hydroxide (1 N, 50 mL). The organic layer was separated, dried ($MgSO_4$), and concentrated to afford a solid. Trituration of the solid with ethyl acetate followed by concentration afforded a residue. Chromatography (ethyl acetate) of the residue afforded 0.77 g (19%) of the desired product as an oil: ^{1}H NMR ($CDCl_3$) δ 2.26 (s, 3H), 6.27 (s, 1H), 6.86 (d, 1H, J = 7.4 Hz), 7.22−7.38 (m, 6H), 7.46 (d, 2H, J = 8.4 Hz), 7.57 (s, 1H, J = 7.9 Hz), 8.47 (d, 1H, J = 4.7 Hz), 8.56 (d, 1H, J = 1.6 Hz); ^{13}C NMR ($CDCl_3$) δ 14.3, 58.4, 105.2, 119.5, 122.4, 123.3, 127.4, 127.9, 128.8, 132.8, 134.8, 137.0, 140.3, 148.5, 148.7, 152.0. Anal. ($C_{21}H_{18}N_4S$) C, H, N.

***N*-(*p*-Cyanophenyl)-*N*′-[(*R*,*S*)-α-(3-pyridyl)benzyl]guanidinoacetic Acid (24).** A solution of glycine (0.566 g, 7.53 mmol) and NaOH (0.30 g, 7.53 mmol) in water (4 mL) was added to a solution of the isothiourea **26** (0.90 g, 2.51 mmol) in ethanol (40 mL), and the mixture was heated at reflux for 6 h and then concentrated. The residue was dissolved in water (50 mL) and washed with CH_2Cl_2 (50 mL), and the pH of the aqueous phase was adjusted to 6. The aqueous solution was lyophilized, and the solid obtained was triturated with ethanol. Concentration of the ethanolic solution afforded 0.8 g (82%) of the desired product as a yellow solid: mp 126 °C dec; ^{1}H NMR (DMSO-d_6) δ 3.82 (s, 2H), 6.24 (s, 1H), 7.19 and 7.59 (AB quartet, 4H, J = 8.5 Hz), 7.23−7.36 (m, 6H), 7.61 (d, 1H, J = 6.5 Hz), 8.4 (dd, 1H, J = 4.5, 1.1 Hz); ^{13}C NMR (DMSO-d_6) δ 56.2, 58.0, 106.5, 109.1, 122.4, 127.6, 128.3, 129.1, 133.7, 135.6, 136.1, 139.6, 143.0, 149.0, 154.6, 170.9. Anal. Calcd for $C_{22}H_{19}N_6O_2$: MW, 385.1617. Found: 386.1627 (HR-FABMS, M + 1).

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