Synthesis of 6-Anilino-2-thiouracils and Their Inhibition of Human Placenta Iodothyronine Deiodinase

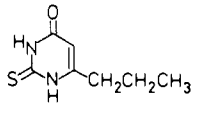
T. Nogimori,† C. H. Emerson,† L. E. Braverman,† C.-F. Wu,† J. Gambino,‡ and G. E. Wright*‡

Division of Endocrinology, Department of Medicine, and Department of Pharmacology, University of Massachusetts Medical School, Worcester, Massachusetts 01605. *Received April 2, 1985*

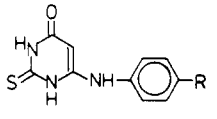
Several 6-anilino-2-thiouracils were synthesized and tested for their ability to inhibit the inner-ring iodothyronine deiodinase from human placenta. The *p*-ethyl and *p*-*n*-butyl analogues were strongly inhibitory to the enzyme and were much more effective than the standard deiodinase inhibitor, 6-propyl-2-thiouracil. The degree of inhibition caused by 6-(*p*-*n*-butylanilino)-2-thiouracil was, moreover, unaffected by high concentrations of reducing agent in the enzyme assay. Attempts to prepare 3-alkyl derivatives via S-debenzylation of 2-benzylthio intermediates led to rearrangement to, for example, 3-methyl-5-benzyl-6-amino-2-thiouracil. This compound also strongly inhibited the deiodinase reaction. Preliminary results suggest that these compounds are useful to study in vitro and in vivo metabolism of thyroid hormones and may be clinically useful to enhance the availability of active thyroid hormones to certain organs.

Rat and human placenta contain an inner-ring iodothyronine 5-deiodinase (PT_4ase) that converts thyroxine (T_4) to the biologically inactive 3,3′,5′-triiodothyronine (reverse T_3, rT_3) and 3,5,3′-triiodothyronine (T_3) to inactive metabolites.[1] In species with hemochorial placentation such as humans and rodents this enzyme may impede the transfer of active thyroid hormones across the placenta. Therefore, inhibition of this enzyme may facilitate the transfer of active thyroid hormones from mother to fetus, a property that could be therapeutically useful in selected clinical situations.

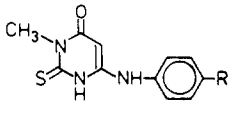
The standard antithyroid drug, 6-propyl-2-thiouracil (PTU), has been reported to inhibit T_3 and rT_3 production by both outer- and inner-ring deiodinases, respectively, from numerous tissues (see, for example, ref 2 and 3 for reviews). Recently, we reported that PTU has weak activity against placenta PT_4ase.[1] Furthermore, its action is readily reversed by reducing agents such as dithiothreitol (DTT) required in assay media to express enzyme activity.[1] We sought, therefore, to identify inhibitors of PT_4ase that would be more potent than PTU and less sensitive to the presence of reducing agents. Among numerous thio heterocyclic compounds available in our laboratory, we found that 6-anilino-2-thiouracil showed modest inhibitory activity against the placenta deiodinase. We, therefore, decided to investigate the synthesis and antideiodinase activity of substituted 6-anilino-2-thiouracils and related compounds and to evaluate their utility in the study of thyroid hormone metabolism.



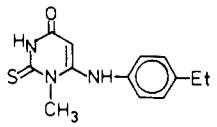
PTU



1, R = H
2, R = Et
3, R = n-Bu



12, R = Et
13, R = n-Bu



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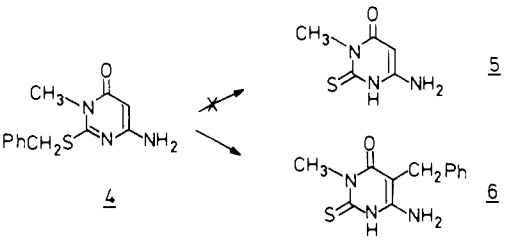
Chemistry. Simple 6-anilino-2-thiouracils, **1–3**, were prepared directly by reaction of 6-amino-2-thiouracil with substituted anilines in the presence of the aniline hydrochloride at high temperature.[4] Among the ring *N*-monoalkyl derivatives, however, only 1-alkyl compounds, e.g. **15**, could be prepared directly. Cyclization of *N*-alkylthioureas with ethyl cyanoacetate gave only 1-alkyl-6-amino-2-thiouracils,[5] suitable as starting materials for compounds like **15**.

In order to prepare 3-alkyl compounds such as **12** and **13**, we attempted to devise a route to the intermediates, 3-alkyl-6-amino-2-thiouracils (e.g., **5**), via S-dealkylation of the corresponding 2-alkylthio compounds. By analogy with S-dealkylations of 3-alkyl-2-(alkylthio)pyrimidones,[6] 2-(methylthio)-3-methyl-6-amino-4-pyrimidone[7] was heated to 140 °C while hydrogen chloride was passed through the molten compound; no products were formed after 2 h of this treatment. Next, debenzylation of 3-methyl-2-(benzylthio)-6-amino-4-pyrimidone (**4**) was attempted. Treatment of this compound with hydrogen chloride at 200 °C led to rearrangement to a product that still contained a benzyl group. The ^1H NMR spectrum of this product in Me_2SO-d_6 showed no 5-H resonance, but peaks at δ 7.35 and 3.60 characteristic of the benzyl group and a single exchangeable resonance at δ 12.0. These data suggested that the compound was 3-methyl-5-benzyl-6-amino-2-thiouracil (**6**). This structure was confirmed through its methylation to a methylthio derivative (**7**) which gave, upon acid hydrolysis, a compound identical with 3-methyl-5-benzyl-6-aminouracil[8] (results not shown).



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The failure to synthesize the intermediate 3-alkyl-6-amino-2-thiouracils by S-dealkylation led to an alternate strategy. The 6-anilino-2-thiouracils **2** and **3** were transformed to the benzylthio derivatives **8** and **9**, and the latter

† Division of Endocrinology, Department of Medicine.
‡ Department of Pharmacology.

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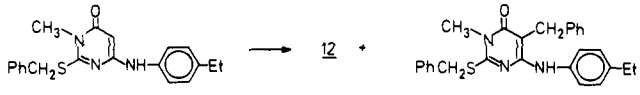
Table I. Inhibition of Human Placenta Inner-Ring Deiodinase Activity by 2-Thiouracils

compd (5 mM)	% inhibn[a]	compd (5 mM)	% inhibn[a]
1	31	15	2
2	85	6	79
3	96	6-amino-2-thiouracil	16
12	0	PTU	29
13	20		

[a] Inner-ring deiodinase (PT_4ase) assay of placenta microsomal fractions employed radioimmunoassay to measure the formation of rT_3 from T_4 in the presence of 0.25 mM DTT (see the Experimental Section). Stock solutions of inhibitors (30 mM) in 0.04 M sodium hydroxide solution were diluted into assay media. 100% (control) activity corresponded to the production of 6.82 pmol of rT_3 per 450-μL assay tube during 60 min at 37 °C. The coefficients of variation for each set of quadruplicate incubations ranged from 5 to 12% (mean ±SD 9 ± 2%).

were methylated to give **10** and **11**. Hydrogen chloride catalyzed debenzylation of **10** and **11** was successful in producing the desired 3-methyl derivatives **12** and **13**. The yields of these products were low, however, partially due to intermolecular capture of the benzyl group by the starting material, leading in the case of **10** to the isolation of the 2-benzylthio 5-benzyl compound (**14**) in 29% yield.



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Biology. The compounds synthesized in this study were tested for their capacity to inhibit the inner-ring deiodinase activity of microsomal fractions of human placenta (see the Experimental Section). The results of Table I demonstrate that the 6-anilino-2-thiouracils **1–3** at 5 mM concentration inhibited the formation of rT_3, the *p*-ethyl (**2**) and *p*-*n*-butyl (**3**) derivatives being considerably more potent than **1**. 1-Methyl (**15**) and 3-methyl (**12**, **13**) derivatives were far less active, but compound **6**, the 5-benzyl rearrangement product, caused 79% inhibition. The standard antithyroid drug PTU, at 5 mM, inhibited PT_4ase by only 29%, whereas 6-amino-2-thiouracil decreased enzyme activity by only 16%. In separate experiments (for example, see below), PTU had variable activity and, indeed, was occasionally inactive under the same assay conditions.

The assay used in the studies reported in Table I employed DTT at a concentration of 0.25 mM, essentially the minimum concentration of reducing agent required for expression of PT_4ase activity.[1] We selected the most active compound, **3**, to test both its potency and its sensitivity to DTT relative to those of PTU. When assayed at 0.25 mM DTT and over a wide concentration range, **3** inhibited PT_4ase in a dose-dependent manner with an apparent K_i of 0.5 mM. Under the same conditions, PTU showed only 12% and 22% inhibition at 1 and 5 mM, respectively (data not shown). When PT_4ase was assayed in the presence of a wide range of DTT concentrations (Table II), compound **3** at 5 mM consistently inhibited activity by 92–95%, whereas 5 mM PTU was essentially inactive. The profound stimulatory effect of DTT on PT_4ase activity is also evident in the results of Table II.

Discussion

The present results indicate that 6-anilino-2-thiouracils may represent a useful new class of thyroid hormone deiodinase inhibitors. With respect to the inner-ring T_4ase from human placenta, which has been employed in this work, compounds such as **1–3** are more potent than the standard inhibitor PTU. Variation of inhibitory activity of **1–3** with substituents (Table I) suggests a strong contribution from hydrophobic binding with enzyme involving the anilino ring, a conclusion consistent with the potent activity of **6**, an analogous compound bearing a hydrophobic 5-benzyl group, and the lack of significant activity of 6-amino-2-thiouracil.

Table II. Inhibition of Human Placenta Inner-Ring Deiodinase Activity by **3** and PTU at Various Concentrations of DTT

[DTT], mM	rT_3 prodn,[a] pmol/tube		
	control	+5 mM PTU	+5 mM **3**
0.2	2.6	2.6	.2
1.56	9.6	13.3	.8
12.5	34.2	43.3	2.5
100	105.7	104.4	4.1

[a] Assays were performed as described in the Experimental Section except that the indicated concentrations of DTT were used. The coefficients of variation for each set of quadruplicate incubations ranged from 2 to 12 % (mean ±SD 5 ± 3%).

The results presented in Table II show that, unlike PTU, the inhibitory activity of **3** is not reversed by an excess of reducing agent in the deiodinase assay. Despite their structural similarities, PTU and the 6-anilino-2-thiouracils may inhibit PT_4ase by different mechanisms; the implications of this result are currently under study.

The placenta inner-ring deiodinase reported here is the one under most active investigation in our laboratory, although both inner- and outer-ring T_4ase activities have been detected in most tissues studied. Consequently, preliminary experiments to examine the scope of activity of this new class of inhibitors have shown that **1–3** are also more potent that PTU in inhibiting the outer-ring deiodinase from rat liver homogenates.[9]

The usefulness of PTU for in vivo studies is limited because it is not only a deiodinase inhibitor but also an antithyroid drug by inhibiting the intrathyroidal synthesis of the thyroid hormones. When administered chronically to rats, 6-anilino-2-thiouracil (**1**) (0.1% in the diet for 12 days, a dose at which no obvious toxicities were apparent) caused strong inhibition of hepatic outer-ring (5′) deiodinase activity but, in contrast to PTU, had no effect on thyroid gland function, since thyroid weight, thyroid ^{131}I uptake, and thyroid organification of ^{131}I were unchanged.[10] Compounds such as **1–3** could ultimately be clinically useful by increasing serum T_4 concentration, thereby enhancing the availability of T_4 to organs such as the brain that contains a PTU-insensitive T_4 outer-ring (5′) deiodinase. This could result in increased generation of T_3, the hormonally active metabolite of T_4, in these organs.[11] Experiments are in progress to determine the influence of 6-anilino-2-thiouracils on the levels of circulating thyroid hormones in rats.

Experimental Section

Melting points were determined on a Mel-temp apparatus and are uncorrected. NMR spectra (60 MHz, ^1H) were taken on a Perkin-Elmer R12B instrument equipped with a TT7 Fourier transform attachment. Chemical shifts are measured in ppm (δ) from internal tetramethylsilane.

Elemental analyses were performed by the Pharmaceuticals Division, Imperial Chemical Industries, Ltd., Alderley Park, England. Results for all new compounds were within 0.4% unless otherwise noted. The synthesis of compound **1** was done as

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described for 2 and 3; it was obtained in 68% yield after crystallization from methanol, mp 281–283 °C, unchanged upon admixture with an authentic sample (supplied by Prof. W. Pfleiderer).

6-(*p*-Ethylanilino)-2-thiouracil (2). A mixture of 6-amino-2-thiouracil (14.7 g, 0.1 mol), *p*-ethylaniline hydrochloride (17.8 g, 0.11 mol), and *p*-ethylaniline (20 g) was heated at 170 °C for 7 h. The cooled mass was mixed with ethanol (25 mL) and water (15 mL). The solid material was filtered and washed with cold ethanol. Crystallization from 90% dimethylformamide gave 16.0 g (63%) of product as a hemisolvate (dimethylformamide), as determined by NMR and elemental analysis; mp 277–279 °C. Crystallization from ethanol gave pure 2. NMR (Me_2SO-d_6) δ 11.80 (s, 1 H, 3-H), 11.43 (s, 1 H, 1-H), 8.04 (s, 1 H, 6-NH), 7.14 δ (s, 4 H, C_6H_4), 4.87 (s, 1 H, 5-H), 2.62 (q, 2 H, CH_2), 1.12 (t, 3 H, CH_3). Anal. ($C_{12}H_{13}N_3OS$) C, H; N: calcd, 16.99; found, 16.3.

6-(*p*-*n*-Butylanilino)-2-thiouracil (3). A mixture of 6-amino-2-thiouracil (1.5 g, 0.01 mol), *p*-*n*-butylaniline hydrochloride (2.1 g, 0.011 mol), and *p*-*n*-butylaniline (2 mL) was heated at 150 °C for 6 h. Ethanol (2.5 mL) and water (1.5 mL) were added, and the slurry was chilled and filtered. The solid was crystallized from aqueous ethanol to give 1.4 g (50%) of 3 as colorless crystals: mp 257–260 °C; NMR (Me_2SO-d_6) δ 11.84 (s, 1 H 3-H), 11.46 (s, 1 H, 1-H), 8.07 (s, 1 H, 6-NH), 7.17 (s, 4 H, C_6H_4), 4.85 (s, 1 H, 5-H), 2.50 (t, 2 H, $ArCH_2$), 1.44 (m, 4 H, $(CH_2)_2$), 0.89 (t, 3 H, CH_3). Anal. ($C_{14}H_{17}N_3OS$·0.33H_2O) C, H, N.

2-(Benzylthio)-3-methyl-6-amino-4-pyrimidone (4). 2-(Benzylthio)-6-amino-4-pyrimidone[12] (2.5 g, 10.7 mmol) was dissolved in a solution of potassium hydroxide (1.3 g, 21.4 mmol) in ethanol (25 mL). To this refluxing solution was added a solution of methyl iodide (3.0 g, 21.4 mmol) in ethanol (5 mL) during 5 min. After 2 h at reflux, the solvent was evaporated and the residue was crystallized from 90% ethanol to give 1.8 g (69%) of 4. An analytical sample was crystallized from ethanol: mp 208–211 °C; NMR (Me_2SO-d_6) δ 7.50 (s, 5 H, C_6H_5), 6.60 (s, 2 H, NH_2), 5.05 (s, 1 H, 5-H), 4.48 (s, 2 H, CH_2), 3.25 (s, 3 H, CH_3). Anal. ($C_{12}H_{13}N_3OS$) C, H, N.

3-Methyl-5-benzyl-6-amino-2-thiouracil (6). Compound 4 (492 mg, 2.03 mmol) was heated to a melt (>200 °C) and treated with hydrogen chloride gas for 30 min. The cooled mass was dissolved in ethanol (30 mL) and diluted with water (15 mL). The precipitated was collected and crystallized from 80% ethanol to give 180 mg (37%) of 6: mp 263–266 °C; NMR (Me_2SO-d_6) δ 12.00 (s, 1 H, 1-H), 7.35 (s, 5 H C_6H_5), 6.40 (s, 2 H, NH_2), 3.60 (s, 2 H, CH_2), 3.55 (s, 3 H, CH_3). Anal. ($C_{12}H_{13}N_3OS$) C, H, N.

2-(Methylthio)-3-methyl-5-benzyl-6-aminouracil (7). A solution of 6 (150 mg, 0.62 mmol) in sodium hydroxide (3 mL) was stirred with methyl iodide (175 mg, 1.24 mmol) for 30 min. The suspension was chilled, and the collected product was crystallized from ethanol, giving 122 mg (77%) of product: mp 159–160 °C; NMR (Me_2SO-d_6) δ 7.3 (s, 5 H, C_6H_5), 6.30 (s, 2 H, NH_2), 3.65 (s, 2 H, CH_2), 3.30 (s, 3 H, 3-CH_3), 2.58 (s, 3 H, S-CH_3). Anal. ($C_{13}H_{15}N_3OS$) C, H, N.

2-(Benzylthio)-6-(*p*-ethylanilino)-4-pyrimidone (8). A solution of benzyl bromide (1.4 g, 8.1 mmol) in ethanol (10 mL) was added during 15 min to a refluxing solution of 2 (1.0 g, 4.05 mmol) and potassium hydroxide (0.23 g, 4.05 mmol) in ethanol (15 mL). After 2 h the cooled suspension was filtered to remove potassium bromide, the filtrate was evaporated, and benzene (30 mL) was added to the residue. The colorless solid was filtered and crystallized from 90% acetic acid to give 0.8 g (57%) of product: mp 240–241 °C; NMR (Me_2SO-d_6) δ 11.81 (s, 1 H, 3-H), 9.04 (s, 1 H, 6-NH), 7.31 (s, 5 H, C_6H_5), 5.31 (s, 1 H, 5-H), 4.38 (s, 2 H, SCH_2). Anal. ($C_{19}H_{19}N_3OS$) C, H, N.

2-(Benzylthio)-6-(*p*-*n*-butylanilino)-4-pyrimidone (9). This compound, made identically to 8, was isolated in 67% yield (from 80% ethanol): mp 224–225 °C. Anal. ($C_{21}H_{23}N_3OS$) C, H, N.

2-(Benzylthio)-3-methyl-6-(*p*-ethylanilino)-4-pyrimidone (10). A solution of methyl iodide (0.8 g, 4.75 mmol) in ethanol (10 mL) was added during 10 min to a solution of 8 (0.8 g, 2.46 mmol) and potassium hydroxide (0.3 g, 4.75 mmol) in ethanol (15 mL). After 4 h at reflux, the solvent was removed and the residue was mixed with chloroform (20 mL). The suspension was filtered and the filtrate evaporated to leave a colorless solid. Crystallization from 90% ethanol gave 0.65 g (81%) of 10: mp 145–147 °C; NMR ($CDCl_3$) characteristic resonances at δ 6.5 (s, 1 H, 6-NH), 5.55 (s, 1 H, 5-H), 4.45 (s, 2 H, SCH_2), 3.35 (s, 3 H, 3-CH_3). Anal. ($C_{20}H_{21}N_3OS$) C, H, N.

2-(Benzylthio)-3-methyl-6-(*p*-*n*-butylanilino)-4-pyrimidone (11). By the same method as described for the synthesis of 10, this compound was isolated in 57% yield (from 90% ethanol); mp 148–149 °C. Anal. ($C_{22}H_{25}N_3OS$) C, H, N.

Reaction of 10 with Hydrogen Chloride. Compound 10 (60 mg, 0.17 mmol) was heated to 160 °C, and hydrogen chloride gas was passed through the melt for 1 h. TLC analysis (EtOAc–$CHCl_3$ (1:1); silica gel) of the solidified mass showed three components, one of which (ca. 25%) appeared to be starting material. The powdered mass was stirred with chloroform (10 mL), and the insoluble colorless material was filtered and crystallized from 90% acetic acid to give 15 mg (27%) of 3-methyl-6-(*p*-ethylanilino)-2-thiouracil (12): mp 311–314 °C; NMR (Me_2SO-d_6) δ 11.7 (s, 1 H, 1-H), 8.11 (s, 1 H, 6-NH), 7.17 (s, 4 H, C_6H_4), 4.98 (s, 1 H, 5-H), 3.42 (s, 3 H, 3-CH_3), 2.5 (q, 2 H, CH_2), 1.13 (t, 3 H, CCH_3). Anal. ($C_{13}H_{15}N_3OS$) C, H, N.

The filtrate remaining after isolation of 12 was concentrated, and the components were purified by chromatography on a column of silica gel. Elution with hexane–chloroform (3:1) gave a colorless solid that, after crystallization from 90% ethanol, afforded 22 mg (29%) of 2-(benzylthio)-3-methyl-5-benzyl-6-(*p*-ethylanilino)-4-pyrimidone (14): mp 127–129 °C; NMR (Me_2SO-d_6) δ 8.30 (s, 1 H, 6-NH), 7.23 (br s, 14 H, 2 C_6H_5, C_6H_4), 4.31 (s, 2 H, SCH_2), 3.89 (s, 2 H, 5-CH_2), 3.36 (s, 3 H, 3-CH_3), 2.50 (q, 2 H, CH_2), 1.12 (t, 3 H, CCH_3). Anal. ($C_{27}H_{27}N_3OS$·0.25H_2O) C, H, N.

Reaction of 11 with Hydrogen Chloride. Compound 11 (102 mg, 0.28 mmol) was heated to 165 °C, and hydrogen chloride gas was passed through the melt for 1 h. TLC of the glassy residue showed a major product, two minor ones, and some starting material. The residue was dissolved in chloroform and chromatographed on a silica gel column. After removal of minor components in cyclohexane–chloroform, the desired 3-methyl-6-(*p*-*n*-butylanilino)-2-thiouracil (13) was eluted with chloroform. The product (27 mg, 40%) was crystallized from ethanol; mp 275–278 °C. The NMR spectrum of 13 was fully commensurate with its proposed structure. Anal. ($C_{15}H_{19}N_3OS$·0.25H_2O) C, H, N.

1-Methyl-6-(*p*-ethylanilino)-2-thiouracil (15). A stirred mixture of 1-methyl-6-amino-2-thiouracil[5] (1.0 g, 6.4 mmol), *p*-ethylaniline hydrochloride (1.1 g, 7 mmol), and *p*-ethylaniline (5 mL) was heated under nitrogen at 150 °C for 5 h. The cooled mixture was heated with ethanol (8 mL), and unreacted 1-methyl-6-amino-2-thiouracil (0.3 g) was removed by filtration. The filtrate was diluted with water (5 mL), and after chilling, 1.1 g of precipitate, containing both starting material and product, was obtained. Crystallization of this solid from ethanol gave 0.6 g (35%) of 15: mp 216–219 °C; NMR (Me_2SO-d_6) δ 12.02 (s, 1 H, 3-H), 8.60 (s, 1 H, 6-NH), 7.21 (s, 4 H, C_6H_4), 4.69 (s, 1 H, 5-H), 3.86 (s, 3 H, 1-CH_3), 2.57 (q, 2 H, CH_2), 1.19 (t, 3 H, CCH_3). Anal. ($C_{13}H_{15}N_3OS$·0.25H_2O) C, H, N.

Isolation and Assay of Placenta Inner Ring Deiodinase. The preparation of human placenta microsomal fractions has been described.[1] The placenta microsomal fraction (225 μg/assay tube) was incubated for 60 min at 37 °C with 290 pmol of T_4 in 0.1 M potassium phosphate buffer–0.01 M EDTA (pH 7.4) in the presence of 0.25 mM dithiothreitol (DTT) in a final volume of 450 μL. Production of rT_3 was determined by radioimmunoassay as described previously;[1] control activity corresponded to the formation of 6.82 pmol of rT_3 per assay tube during 60 min at 37 °C.

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