Synthesis of (6-*R*,*S*)5-CH_3-H_4PteGlu$_n$ from PteGlu$_n$

References: Yeo, E. J., and Wagner, C. (1992) Purification and properties of pancreatic glycine N-methyltransferase. *J Biol Chem* 267, 24669-24674; Nando Todari, A., and Marazza, N. F. (2000) Process for the reduction of pterins. (US Patent 6162914) Cerbios-Pharma S.A., Switzerland

Outline: This method uses $NaBH_4$ to reduce folic acid (PteGlu$_n$) to H_4PteGlu$_n$ with the modification of the water-soluble $Pb(NO_3)_2$ to catalyze the reduction. The inclusion of $Pb(NO_3)_2$ allows less $NaBH_4$ to be used. The H_4PteGlu$_n$ is then condensed with HCHO to produce 5,10-methylene-THF (CH_2-H_4PteGlu$_n$), which is then reduced with $NaBH_4$ to 5-CH_3-H_4PteGlu$_n$.

Method:

1. Weigh 40 μmol PteGlu$_n$ (20 – 40 mg depending on # of glutamates) into a 2 ml graduated free-standing screwcap tube with round bottom. Add 200 μl $Pb(NO_3)_2$ (0.265 mM) prepared as follows:

    a. dissolve 20-30 mg $Pb(NO_3)_2$ (Mallinkrodt) in ddH_2O to 8.8 mg/ml = 26.5 mM (use 1-2 μl conc HCl to dissolve completely)

    b. dilute 1:100 with 10 mM Tris-Cl, pH 7.5 to make 0.265 mM working stock

Adjust pH to 7.5 with 5 N NaOH (15-35 μl); spot small amounts on pH paper to check) and vortex to dissolve PteGlu$_n$ (should get clear, dark yellow solution)(higher polyglu require considerable vortexing and more NaOH to dissolve completely). Check spectrum of 10^{-4} dilution in 10 mM Tris-Cl, pH 7.5 (see figure 1).

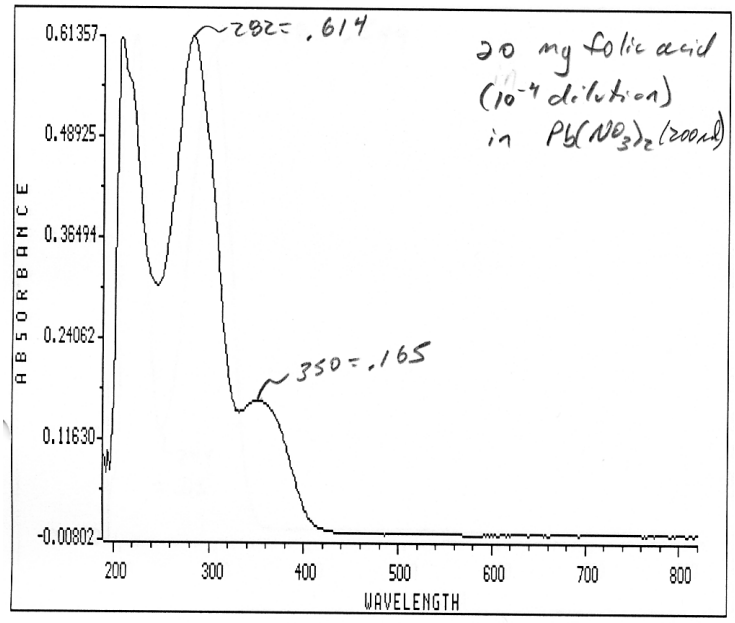
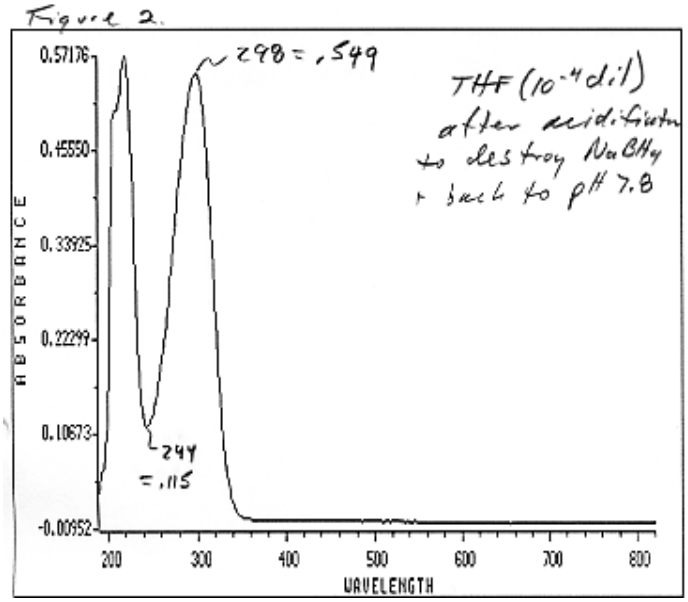


Fig. 1

2. Add stir bar and move to cold room. Use tube rack to hold tube while stirring at top speed. Add 5 mg $NaBH_4$ (Sigma) (132 μmol) in 25 μl ddH_2O over 20 min (5 X 5 μl aliquots). Maintain pH at 8.5-8.8 with 20% (w/v) citric acid (2-5 μl per addition of $NaBH_4$). Once all $NaBH_4$ is added, move to room temp, flush tube with argon (or N_2), and stir at top speed in dark for 2 hrs.

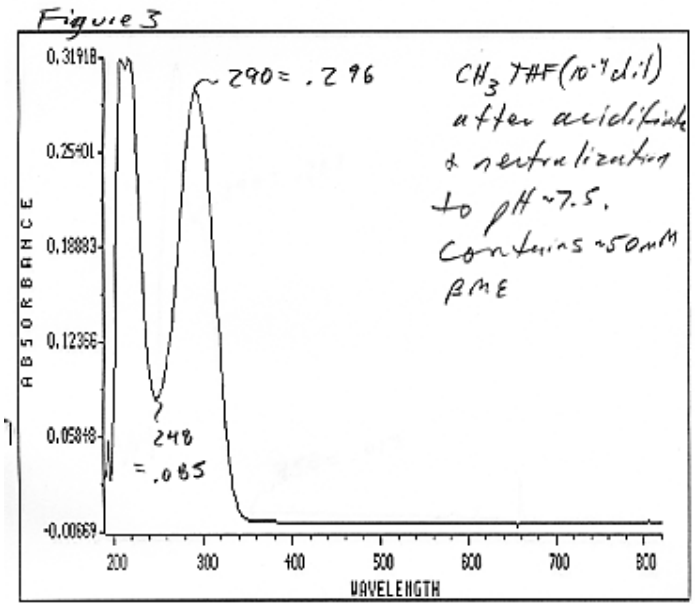
3. Cool to 4°C, lower pH to 5.0 with 5 N HOAc (~20 μl) to destroy excess $NaBH_4$ (some bubbling will occur). Bring pH back to 7.8 with 5 N NaOH. Product should be $H_4PteGlu_n$. Check spectrum of 10^{-4} dilution (see figure 2).



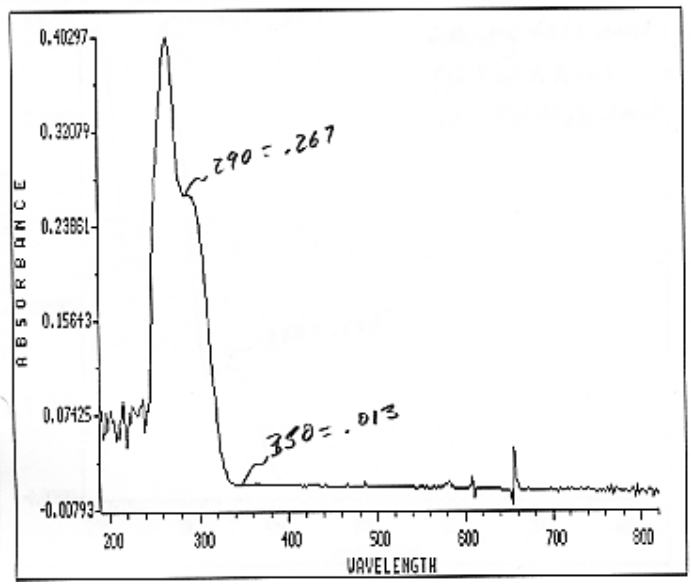
4. Add 6.4 μl (80 μmol) HCHO (37% soln), flush tube with argon, and incubate at 45°C, 15 min in dark to convert $H_4PteGlu_n$ to CH_2-$H_4PteGlu_n$.

5. Add 10 mg $NaBH_4$ (264 μmol) in 50 μl ddH_2O over 20 min (5 X 10 μl aliquots). Maintain pH at 8.5-8.8 with 20% (w/v) citric acid as before. Once all $NaBH_4$ is added, flush tube with argon and incubate at 45°C, 60 min in dark to reduce to 5-CH_3-$H_4PteGlu_n$.

6. Remove stir bar, cool to 4°C, add 2-mercaptoethanol to 50 mM (~1.4 μl of 14.3 M stock). Adjust pH to 5.0 with 5 N HOAc to destroy excess $NaBH_4$ (vortex till no more bubbling). Bring back to ~7.5 with 5N NaOH. Check spectrum of 10^{-4} dilution (see figure 3). Store under argon (or N_2) in dark at –20°C.



Notes: Starting material ($PteGlu_n$) has maxima at 282 (E = 23.4 mM^{-1}) and 350 nm (Fig. 1). $H_4PteGlu_n$ has a maximum at 298 nm (E = 30 mM^{-1}), and a min at 244 nm (Fig. 2). The final product (5-CH_3-$H_4PteGlu_n$) has a max at 292 nm (E = 31.7 mM^{-1}) and a min at 248 nm (Fig. 3). With $PteGlu_1$, this procedure gave ~93% yield, and the final product had less than 3% $H_4PteGlu_n$ based on its reaction with 5N HCl/60% formate:



Diluted 2 μl of product into 200 μl Tris, and then 10 μl of this into 400 μl Tris (7.5). Add 100 μl 5N HCl/60% formate, heat at 80°C, 10 min, cool to RT and read (blank against buffer + HCl/formate). A peak at 350 nm is diagnostic of THF; lack of peak indicates CH_3-THF.