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A Naturally Occurring Pyrimidodiazepine in *Drosophila*: Chemical and Spectral Properties and Relationship to Drosopterin†

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ABSTRACT: The structure of an intermediate, in drosopterin biosynthesis, as 6-acetylpyrimidodiazepine has been confirmed by high-resolution mass spectra, ^{13}C NMR, chemical ionization mass spectra, and chemical properties. A trivial name of 6-acetylhomopterin is suggested and should replace the term "quench spot" used heretofore. The structure of drosopterin includes, in part, a pyrimidodiazepine, a compound that consists of a fused six- and seven-membered heterocyclic ring system. Earlier studies demonstrated that 6-acetylhomopterin strongly stimulated the enzymatic synthesis of drosopterin and related eye pigments by preparations from *Drosophila*. The occurrence in nature is quite limited for diazepines; drosopterin and homopterin are the first examples in eukaryotes.

The regulation of the synthesis of the red drosopterin eye pigments of *Drosophila* is being examined from biochemical and genetic viewpoints. The concentrations of the drosopterins and of a number of simple pterin derivatives in the heads of several mutants have been determined (Wilson & Jacobson, 1977). In the course of this study a substance was found that had properties not typical of pteridines but affected by the same mutations that affect the drosopterins. This substance was termed quench spot (QS)[1] since, in contrast to the pteridines, it failed to fluoresce under certain circumstances. Subsequently quench spot was found to be an apparent intermediate in the enzymatic biosynthesis of pteridines (Dorsett & Jacobson, 1982). Also, when Wiederrecht et al. (1981) were studying drosopterin biosynthesis, they noted that a heat-stable stimulatory factor is present in extracts of *Drosophila* heads. They isolated this factor and proposed that its structure was that of a diazepine ring fused to a pyrimidine, i.e., a pyrimidodiazepine.

Seven-membered rings such as the tropolones and diazepines are uncommon natural products. Diazepines have been obtained from a few microorganisms (Suhadolnik, 1979; Oakami, 1973), but among animals the only diazepines reported are those in *Drosophila*. One is the pyrimidodiazepine (Wiederrecht et al., 1981); the other is drosopterin (Theobold & Pfleiderer, 1978).

This report concerns the nature of quench spot, offers evidence for the pyrimidodiazepine structure, beyond those presented by Wiederrecht et al. (1981), and presents some of its properties.

Materials and Methods

Chromatography media were obtained as follows: CM-23 and CM-52, forms of carboxymethylcellulose, and cellulose (CC-31) were from Whatman. The Zorbax C8 reversed-phase column (0.46 × 25 cm) was from Du Pont. HPLC-grade methanol came from Burdick and Jackson or Fisher Scientific. The C_{18} Sep-PAK was from Waters Associates. Thin-layer cellulose sheets (No. 13255) were from Eastman Kodak.

The vacuum cleaner was a "Kenmore Hand Vac" (Model 208, 61110, from Sears, Roebuck and Co.).

The reagent gases for chemical-ionization mass spectrometry were obtained from the following sources: isobutane from Matheson Gas Products, Inc. (East Rutherford, NJ); D_2O (>98.5 atom % D) from Aldrich Chemical Co. (Milwaukee, WI); ND_3 (>99 atom % D from Merck and Co. (St. Louis, MO). *N,O*-Bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane was purchased from Pierce Chemical Co. (Rockford, IL).

Mass Spectrometry. Electron-ionization mass spectra were recorded by using a Varian MAT 731 mass spectrometer, with samples introduced by direct probe (ionizing energy 70 eV, ion source temperature 200 °C). Low-resolution mass spectra were acquired by magnetic scanning; high-resolution spectra were recorded photographically at a resolution of 16 000 by using Ionomet-evaporated AgBr plates. Standard mass com-

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[1] Abbreviation: QS, quench spot.

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position calculations were done with a Varian SS100C data system by using data derived from a Gaetner M1205PC comparator. The chemical ionization (isotopic exchange) mass spectra were obtained with a Varian MAT 1125 instrument under standard chemical ionization conditions; the ion source temperature was 200 °C and the pressure approximately 0.5 torr; sample introduction was by direct probe.

Preparation of Trimethylsilyl Derivatives. To approximately 25 μg of dried quench spot was added 5 μL of dry dimethylformamide and 20 μL of *N,O*-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane. The mixture was heated for 1 h at 100 °C in a sealed tube.

^{13}C NMR Spectra. The ^{13}C NMR spectra were obtained at 22.50 MHz by using a JEOL FX90Q Fourier transform equipped with a broad-band synthesizer. The spectra were run at an ambient probe temperature of 23 °C and with an internal deuterium lock. Each sample was dissolved in 50 μL of [^{12}C,^{2}H]dimethyl sulfoxide (Prochem, Summit, NJ) and transferred to a 1.7-mm spinning tube, which was then sealed. Because of the low solubility of the compounds studied, tetramethylsilane (Me_4Si) was not added to each sample. Instead, the chemical shift of a peak relative to the solvent peak, δ_{Me_2SO}, was added to the displacement of the solvent peak from internal Me_4Si to yield the chemical shift relative to Me_4Si, δ_{Me_4Si}.

$$\delta_{Me_4Si} = \delta_{Me_2SO} + 39.71 \text{ ppm}$$

The proton-decoupled spectrum was obtained by using 20K pulses with a 6.5-μs (45°) pulse angle and a 5-s pulse delay. The proton coupled spectra was acquired under similar conditions except that 47K pulses were accumulated with a 6-s pulse delay. A decoupling technique (NOE mode) was used to suppress the decoupling current during signal acquisition.

^{1}H NMR Spectra. The proton spectra were obtained at 250 MHz by using a Bruker WM-250 Fourier transform spectrometer equipped with a broad-band synthesizer with a pulse width of 2 μs and a total of 500 pulses with a 50-s pulse delay. The probe temperature was 26 °C and the spectra were acquired by an internal deuterium lock. The sample was dissolved in 0.5 mL of [$^{2}H_6$]Me_2SO and measured in a 5-mm tube.

High-Performance Liquid Chromatography (HPLC). Fukushima & Nixon (1979a) described conditions for chromatographic separation of several pterin derivatives on C_{18} reversed-phase columns. A reversed-phase column (0.46 × 25 cm) of C8 Zorbax (Du Pont) was maintained at 35 °C and equilibrated and developed isocratically with 15% methanol containing 10 mM ammonium phosphate (pH 7.4) with a flow rate of 2 mL/min. The pump, the absorbance monitor at 260 nm, and the fluorescence monitor (excite at 360 nm, emit at >418 nm) were described earlier (Dorsett & Jacobson, 1982).

Oxidation Reduction and Derivatization Reactions. (*1*) *$KMnO_4$.* Quench spot (QS) was dissolved in aqueous solution (A_{384} = 2.2) that contained 14 mM $KMnO_4$ and 14 mM NaOH in a capped polypropylene tube and incubated at 42 °C at 60 min in the dark. Aliquots of 1% sodium ascorbate were added until the color of $KMnO_4$ disappeared; the MnO_2 formed was removed by centrifugation, and 10 μL of the supernatant was analyzed by HPLC. Sepiapterin was oxidized in the same manner; a reagent control was also analyzed. This procedure degrades the side chain on the C-6 position of pterin to produce pterin-6-COOH (Kaufman, 1963).

(*2*) *$NaIO_4$.* QS was dissolved in aqueous solution (A_{384} = 1.2) that contained 16 mM $NaIO_4$; the tube was capped and incubated at 25 °C in the dark for times up to 30 min. The product (10 μL) was analyzed by HPLC. Sepiapterin in water replaced QS in similar reactions. This reagent oxidizes *cis*-hydroxyl groups on the side chain.

(*3*) *2,4-Dinitrophenylhydrazine.* An aliquot (15 μL) of this reagent (saturated solution in concentrated H_2SO_4) was added to H_2O (20 μL) and 95% ethanol (70 μL); the precipitate was removed by centrifugation. Aliquots of QS (20 μL, A_{384} = 3.1) and KOH to keep the pH >2 were added to 2 μL of the supernatant and incubated at 42 °C for 20 min in the dark to produce a colored product that is indicative of the presence of a carbonyl.

(*4*) *$NaBH_4$.* Reduction was accomplished in 0.01 M $NaBH_4$/0.01 N NaOH for 15 min in the dark at 25 °C.

(*5*) *Iodine.* The procedure of Fukushima & Nixon (1979b) was employed as follows. QS was dissolved in an aqueous solution (A_{384} = 2.2) to which was added, if needed, 0.01 N HCl or 0.01 M KOAc (pH 5.0) and $^1/_{12}$ volume of the iodine solution (1% I_2 in 2% KI). After 30 min in the dark at 25 °C aliquots of 1% ascorbate were added to discharge the iodine color, and aliquots were analyzed by HPLC. Treatment of the iodine oxidation products with 2,4-dinitrophenylhydrazine, $NaBH_4$, or $NaIO_4$ was performed as described above after the addition of the ascorbate.

Drosophila Production. So that adult *Drosophila* could be produced in batches of 100–200 g, Lucite boxes (40 × 50 × 70 cm) were used; they have a 25 × 55 cm opening to which is attached a cloth sleeve with an elastic cuff to allow introduction and removal of objects without allowing the escape of the resident flies. Twelve trays (13 × 17.5 × 2.4 cm, Mobile Foam 4D), commonly used in food markets, were each filled with 400–500 mL of media (Lewis, 1960) and placed on racks of plastic-coated wire after adding a 1–2-mm layer of dried bakers' yeast and moistening it with water. Adult *Drosophila* of ages 1–7 days (8–15 g) were allowed to lay eggs for 4 days at which time they were removed, and bats of absorbent cotton (12 × 15 cm) were laid on each tray to which the pupae could attach. Once adults began emerging (10 days at 25 °C) they were harvested dialy and stored at −80 °C. A small vacuum cleaner was used to draw the adults through a vinyl hose into a chamber consisting of a plastic bottle (11.5-cm diameter) that had been cut horizontally in half and reassembled with cheesecloth stretched across the open end of the top half. The diameter is sufficient to allow the adult *Drosophila* to decelerate from the rapid flow up the hose (2.4-cm diameter) and to achieve ~90% survival. Adults collected in this manner can be used to inoculate other growth boxes to produce a subsequent generation.

Purification of QS. To a 1-gal plastic jug containing 50 g of adult flies was added liquid nitrogen. As soon as the nitrogen had evaporated, the jug was stoppered with a foam plug and shaken vigorously for 2–3 min to dismember the flies. The contents were then poured into the upper of two stainless steel sieves that had been chilled to −80 °C. The upper sieve was 24 mesh (710-μm opening) and retained the abdomen–thorax; the lower one was 35 mesh (425-μm opening) and retained the heads, allowing the wings and legs to pass through. These sieves were shaken at 4 °C on a mechanical shaker for 1 min. The yield of heads was 5–5.5 g.

The previous procedure for purification of QS (Dorsett & Jacobson, 1982) was modified to allow double the amount of heads to be used. Thirty grams of heads was homogenized in a Virtis blender at a setting of 70 with 150 mL of a 1:1 mixture of 2-propanol and 1% aqueous mercaptoethanol. The homogenate was centrifuged at 10000*g* for 5 min at 25 °C. The pellet was rehomogenized in an additional 150 mL. The supernatants were combined, and the volume was reduced to

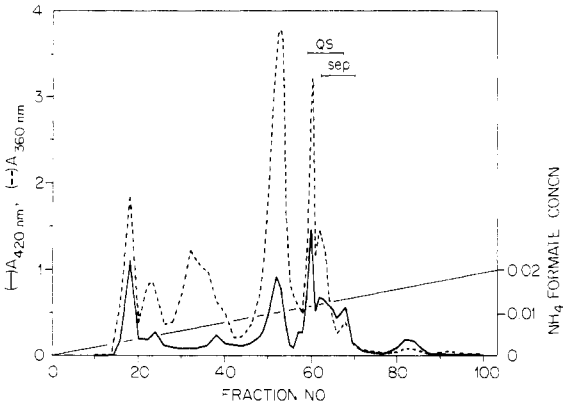

FIGURE 1: Chromatography of QS on carboxymethylcellulose (CM-52). The QS-containing fractions from the CM-23 column were pooled, lyophilized, dissolved in water, and added to the CM-52 column. QS eluted in fractions 58–67 and sepiapterin in 62–70. Fractions contained 20 mL.

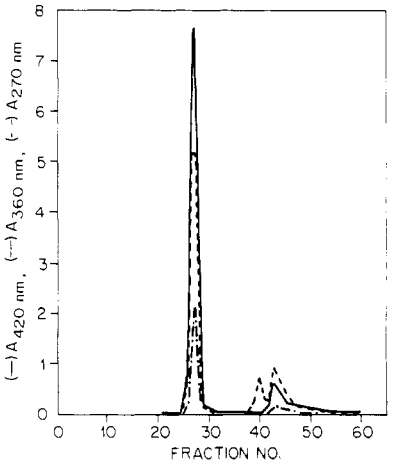

FIGURE 2: Chromatography of QS on cellulose. The QS- and sepiapterin-containing fractions from the CM-52 column were pooled, lyophilized, and dissolved in 5 mM $NiCl_2$/0.56 M NH_4Cl, pH 5.8, and after adding the sample, it was eluted with the same solution.

100–130 mL by flash evaporation at 50 °C and centrifuged. All operations were carried out at 25 °C under low-intensity red light unless specified otherwise.

The supernatant was chromatographed at 4 °C on a column (8 × 18 cm) of CM-cellulose (CM-23), ammonium form, at a flow rate of 2 mL/min by using water to elute the sample. The fractions (20 mL), containing QS along with sepiapterin, were identified by applying 20 μL to a cellulose thin-layer sheet and developing the sheet with 3% NH_4Cl for 30–40 min. QS migrates ahead of sepiapterin and appears as a dark spot under 360-nm light when the sheet is wet if a large amount of QS is present. Lower amounts were readily detected with 360-nm light by immersing the dry sheet in liquid nitrogen and observing a greenish yellow fluorescence that does not continue (phosphoresce) when the light is removed. The pooled fractions were lyophilized, dissolved in 20–30 mL of H_2O (warming to 50 °C facilitates dissolution) and applied to a CM-microcrystalline cellulose (CM-52) column (2.5 × 84 cm), previously washed with 0.5 M ammonium formate and then H_2O prior to pouring into the column. The sample was eluted at 4 °C with a 2000-mL linear gradient from 0 to 20 mM ammonium formate at a flow rate of 0.5 mL/min in 20-mL fractions.

QS can be detected by thin-layer chromatography (above) or by measuring absorbance at 360, 380, and 420 nm. The 380/360 and 420/360 ratios are 1.14 and 0.44, respectively. Resolution from sepiapterin is usually incomplete (Figure 1). The leading two to three fractions of the QS zone usually contained fine needle-shaped crystals of this compound. Those fractions were pooled and found to be essentially free of other absorbing and fluorescing substances as judged by TLC and HPLC. The remaining fractions that contained QS and sepiapterin together were rechromatographed on CM-52 in the same manner. So that the QS or sepiapterin could be concentrated, 5 mL of a QS solution (A_{380} = 2.2) was applied to a C_{18} Sep-PAK. After three rinses with 5 mL of H_2O the substance was eluted with 1.5 mL of methanol and the methanol removed by flash evaporation. The sample was dissolved in water and lyophilized to give a greenish yellow powder that was stored at −20 °C. Sometimes the powder was orange but on dissolving in water it gave a greenish yellow solution that is typical of QS.

Recently a third column procedure has been added that resolves QS and sepiapterin completely. All fractions from the CM-52 column that contained QS were pooled, lyophilized, dissolved in 20–30 mL of 5 mM $NiCl_2$/0.56 M NH_4Cl, and applied to a cellulose column (2.5 × 84 cm) that was equilibrated in 5 mM $NiCl_2$/0.56 M NH_4Cl adjusted to pH 5.8. The sample was eluted in the dark at 25 °C with the same solution at a flow rate of 0.7 mL/min in 13.7-mL fractions (Figure 2). The $NiCl_2$ stabilizes QS and improves the resolution from sepiapterin as compared to NH_4Cl alone; QS is completely degraded in 36 h in 0.56 M NH_4Cl (pH 4) in the absence of $NiCl_2$. The adsorption of QS on the C_{18} Sep-PAK was ineffective unless 10 mM EDTA was added to complex the Ni^{2+}; sepiapterin adsorbed in the presence or absence of EDTA. Sepiapterin, in contrast to QS, is not degraded by 0.56 M NH_4Cl.

Purification of Sepiapterin. After elution from the cellulose column the fractions containing sepiapterin were pooled and collected by the Sep-PAK procedure to remove the $NiCl_2$ and NH_4Cl, and the methanol was removed by flash evaporation. The sepiapterin was dissolved in water and chromatographed on a C8 reversed-phase column by using 10% methanol for isocratic elution. The fractions containing sepiapterin were lyophilized and the dry orange powder was stored at −20 °C.

Results

Chemical Reactivity. Since both QS and sepiapterin apparently are derived from a common precursor (Dorsett & Jacobson, 1982), the properties of these two substances were compared.

Oxidation by $KMnO_4$ converted sepiapterin to pterin-6-COOH (identified by HPLC). On the other hand, for QS the color and the characteristic HPLC peak of QS were lost and no pterin-6-COOH or other A_{260}-absorbing products were detected other than that due to the unadsorbed reagent. Therefore, QS is degraded by $KMnO_4$ but the product is not typical of a substituted pterin.

By oxidation with $NaIO_4$ sepiapterin was converted to 6-carboxypterin but QS was not. Again the color and HPLC peak of QS were lost and no new A_{260}-absorbing peaks were produced. Thus the ring system of QS is apparently degraded by this reagent.

Addition of 2,4-dinitrophenylhydrazine to QS resulted in the appearance of a red-orange color, but the product was not isolated or characterized. The presence of a carbonyl group is likely.

Oxidation of reduced pterins with I_2 usually gives products that are more fluorescent and with altered chromatographic mobility. This was the case for sepiapterin; QS was irreversibly

Table I: Assignment of ^{13}C NMR Spectral Lines

structure	C-2	C-4	C-6	C-7	C-7a	C-7b	C-9	C-10	C-11	C-11a	C-12
II	158.30[a]	161.22	149.85		29.58	39.93	152.56	102.71	197.63		23.02
IV	155.86[a]	158.62	138.20	39.17			156.62	103.42	199.31	66.58	21.24
VII[b]	157.4	162.5	150.0	150.0			155.1	129.2			

[a] In dimethyl sulfoxide; chemical shifts are given with respect to internal tetramethylsilane (see the text). [b] From Ewers et al. (1973).

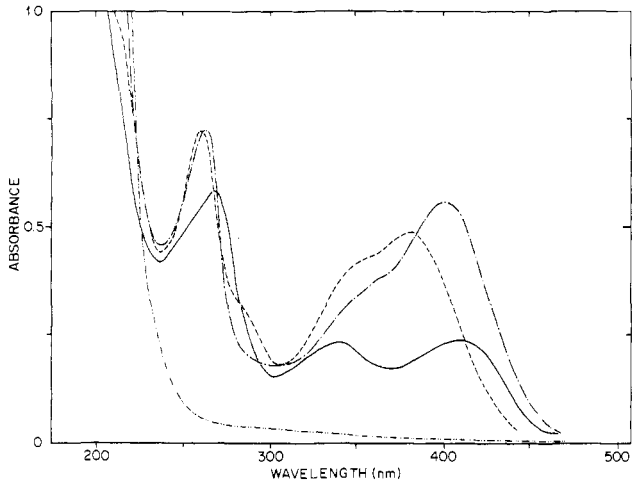

FIGURE 3: Absorption spectrum of quench spot. H_2O (- - -); 0.1 N NaOH (-·-); 0.1 N HCl (—); 0.1 N HCl, hydrogenated (-··-). Hydrogenation was carried out with platinum oxide as the catalyst. Reprinted with permission from Wilson & Jacobson (1977). Copyright 1975 Plenum.

oxidized to produce two A_{260}-absorbing substances, as seen on HPLC, that eluted at approximately three-tenths the volume of QS. As the pH increased from 4 to 7, the amount of the second substance also increased; in the presence of acetate buffer (pH 5.0) neither product was formed to an appreciable extent. Like QS, these products were nonfluorescent as they emerged from the C8 column. From the amounts formed at various times there seemed to be no interconversions. They did not react with 2,4-dinitrophenylhydrazine or $NaBH_4$; only the one eluting earlier was oxidized by periodate. In summary, iodine oxidation caused QS to degrade rather than undergo a simple oxidation typical of pterin derivatives.

Reduction of QS or sepiapterin by $NaBH_4$ occurred in a few minutes. In the case of QS, the reduced product eluted from the C8 column in half the volume required for QS, and it was destroyed by oxidation with either iodine or periodate. This too indicates that QS is undergoing reactions not typical of pterins.

These characteristics indicate that QS probably contains a carbonyl group that may be adjacent to a carbon attached to another oxygen or nitrogen; a ring that can be oxidized and reduced also may be present but it is clearly not a typical pterin derivative.

Ultraviolet Absorption. The spectra of QS are shown in Figure 3. Although the absorption maxima are in regions characteristic of pteridines, they are also located in regions characteristic of tropolones and diazepines for which maxima occur at 250–260, 330–360, and 380 nm (Stern & Timmons, 1970). For determination of the pK_a values of QS, the change in absorption at 405 nm with pH was measured. As shown in Figure 4 the inflection points occur at pH 2.7 and 10.7. However, below pH 2 QS is rapidly and irreversibly degraded. The absorption decreases as QS is degraded, so the lower pK of 2.7 may be in error on the low side.

Molecular Weight. A low-resolution mass spectrum indicated a molecular weight for QS of 221, which is consistent

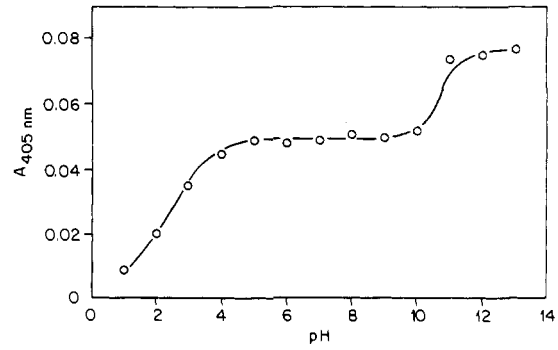

FIGURE 4: Effect of pH on the absorbance at 405 nm of quench spot.

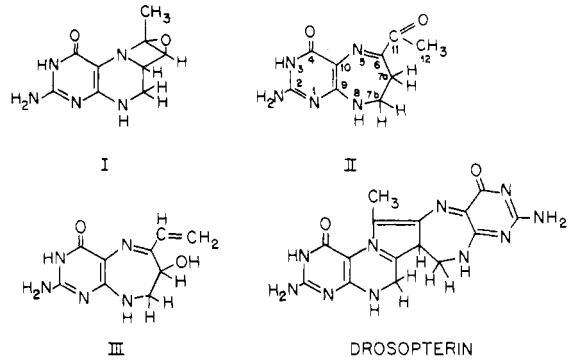

FIGURE 5: Structures of compounds with a molecular weight of 221 considered for QS; also shown is the structure of drosopterin.

with an elemental composition of $C_9H_{11}N_5O_2$. Thus, it was clear that no carbons had been removed during the reactions arising from dihydroneopterin triphosphate. A large number of structures are conceivable, but we consider only those that seem to be reasonable biosynthetic precursors of drosopterin, such as I, II, and III (Figure 5). Structure I is ruled out on the basis of the ultraviolet absorption spectra; the strong absorption at 384 nm is inconsistant with the fully reduced pyrazine ring.

Since the structure of QS can not be unequivocably assigned from the mass spectral data alone, carbon-13 and proton NMR spectra of QS were used to distinguish the remaining two candidates.

^{13}C NMR and ^1H NMR Spectra. Carbon-13 magnetic resonance has often been used to assign structures of nitrogen heterocycles (Pugmire & Grant, 1968, 1971; Thorpe et al., 1974). The proton-decoupled spectra of sepiapterin and QS are given in parts A and B of Figure 6, respectively. The assignment of the lines are given in Table I. From these data, the structure of QS was found to correspond to structure II.

The protonated carbons were assigned by using proton-coupled spectra. The chemical shifts for carbons 7a and 7b in QS (II) are similar to the saturated carbons in benzoheptatriene (V) (Figure 7) (Levy & Nelson, 1972). The presence of the nitrogen adjacent to C-7b shifts it downfield with respect to C-7a. These two carbons were observed to be split in the proton-coupled spectrum. However, because of the low solubility of QS and the resulting low signal to noise ratio of the proton-coupled spectrum, and interference from

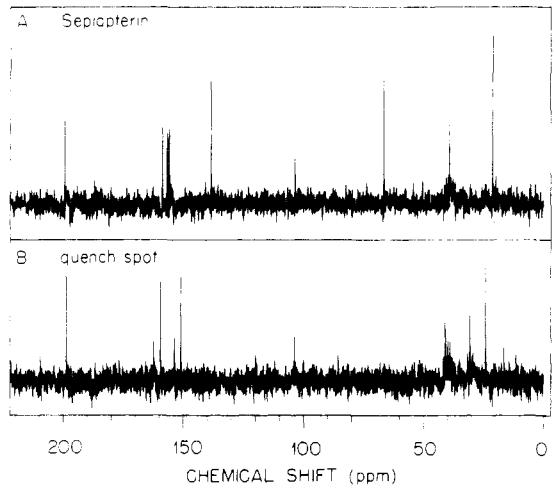

FIGURE 6: ^{13}C NMR spectrum of sepiapterin (A) and quench spot (B).

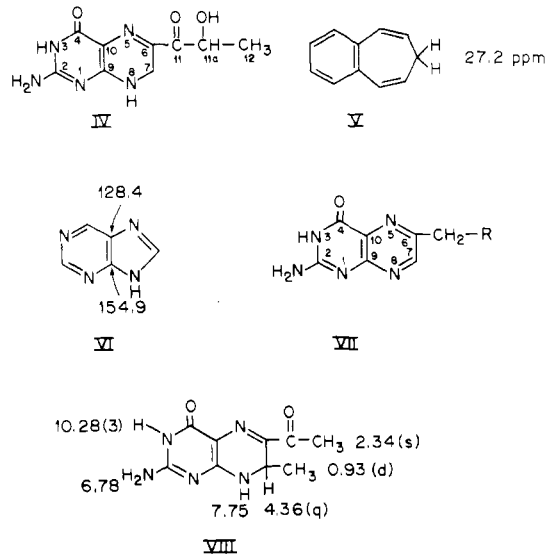

FIGURE 7: Structural assignments of ^{13}C NMR and ^{2}H peaks for QS, sepiapterin, and related compounds.

the solvent, the number of protons attached to these carbons could not be determined. The analogous saturated carbon in sepiapterin, C-7, is assigned similarly. In both QS and sepiapterin, a methyl carbon, C-12, is observed and exhibits a triplet in the coupled spectra ($^{1}J_{CH}$ = 128.2 Hz). Additionally, both molecules also contain a carbonyl carbon (C-11) at approximately 198 ppm, which is typical of carbonyls attached to aromatic rings (Levy & Nelson, 1972). The bridging carbons 9 and 10 are assigned by their chemical shifts. The carbon adjacent to the two nitrogens, C-9, is shifted downfield with respect to C-10, as is seen in the similar molecule (purine) VI (Levy & Nelson, 1972). The presence of the carbonyl on C-4 shifts the resonance of C-10 upfield of the analogous carbon in purine.

The four lines occurring between 138 and 162 ppm corresponding to carbons 2, 4, 6, and 9 cannot be unambiguously assigned in either molecule. Ewers et al. (1973) studied a substituted pteridine, VII, and assigned the carbons with respect to their pH dependence. These assignments are given in Table I. Carbons 2 and 4 in both sepiapterin and QS are thus assigned with respect to the analogous carbons in VII, along with the relative intensities of the spectral lines. The intensity of amine-substituted carbons, like C-2, are usually strong and sharp due to enhanced nuclear Overhauser effects.

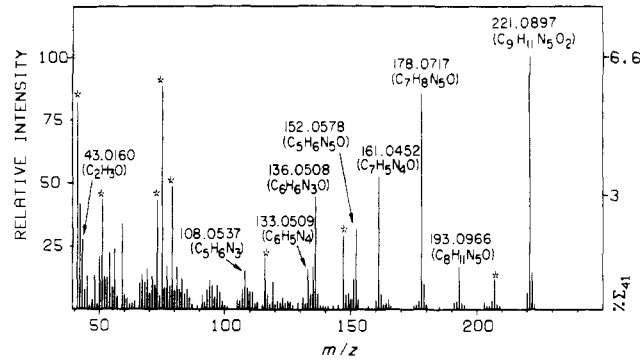

FIGURE 8: Mass spectrum of QS. Peaks denoted by asterisks have major contributions from impurities as determined from selected ion recordings. Experimentally measured exact mass values are as shown.

On the other hand, carbonyls like C-4 tend to be less intense. The assignments for carbons 9 and 6 are also made on the basis of relative intensities, with the bridging quaternary carbon expected to yield a much less enhanced line than the alkyl-substituted quaternary carbon. In addition, the proton-coupled spectrum of QS showed that the carbons at 158.3 and 149.9 ppm are coupled weakly to nearby protons, which also supports their assignments to C-2 and C-6, respectively.

The proton NMR spectrum of QS exhibits six peaks. A sharp singlet at 2.33 ppm (relative to internal tetramethylsilane) corresponds to the three protons on C-12. Two broad triplets centered at 2.78 and 3.18 ppm are assigned to the two protons on C-7a and the two protons on C-7b, respectively. At lower field there are four peaks, which may be assigned to the various amine groups. After deuterium exchange, these four signals disappear, whereas the peaks at 2.33, 2.78, and 3.18 remain unchanged. A broad singlet at 6.51 ppm, corresponding to two protons, is assigned to the amino group on C-2. The signal at 7.56 ppm appears to be a pseudoquartet and is therefore consistent with the proton on C-8 due to the diastereotopic nature of the protons on C-7b. A very broad band appears at 10.3 ppm and is assigned to the proton on C-3, since this position is the most acidic in the molecule. These assignments correspond with those given for 6-acetyl-7-methyl-7,8-dihydropterin (VIII) by Sugiura & Goto (1969).

Mass Spectrometry Studies on Quench Spot. The electron-ionization mass spectrum of quench spot is shown in Figure 8. The figure also shows computer-derived elemental compositions from the corresponding high-resolution mass spectrum, which indicates a molecular composition of $C_9H_{11}N_5O_2$ (calculated 221.0912). The molecular weight was further confirmed by the chemical-ionization mass spectrum, which gave m/z 222 for MH^+; reagent isobutane gas was used.

The major fragmentation pathway of m/z 221 under electron ionization conditions involves loss of $C_2H_3O\cdot$ to generate ion m/z 178. The presence of ion 43.0160 ($C_2H_3O^+$) supports this fragmentation and suggests the presence of a C_2H_3O moiety in the molecule. Loss of $CH_3CO\cdot$ would produce an ion of mass 178.0729, whereas, alternatively, loss of HNCO would yield an ion of mass 178.0855. Since the experimentally measured value is 178.0717, the former pathway is indicated. Other important fragmentation pathways and plausible ion structures, which are also consistent with the structure earlier proposed (Wiederrecht et al., 1981), are shown in Figure 9.

In an effort to establish the number of active hydrogens, chemical-ionization mass spectra were obtained by using D_2O and ND_3 as reagent gases. This technique has previously been used successfully for determining the number of active hydrogens in heterocyclic compounds (Hunt et al., 1972). The ion pattern observed indicates incomplete exchange of up to

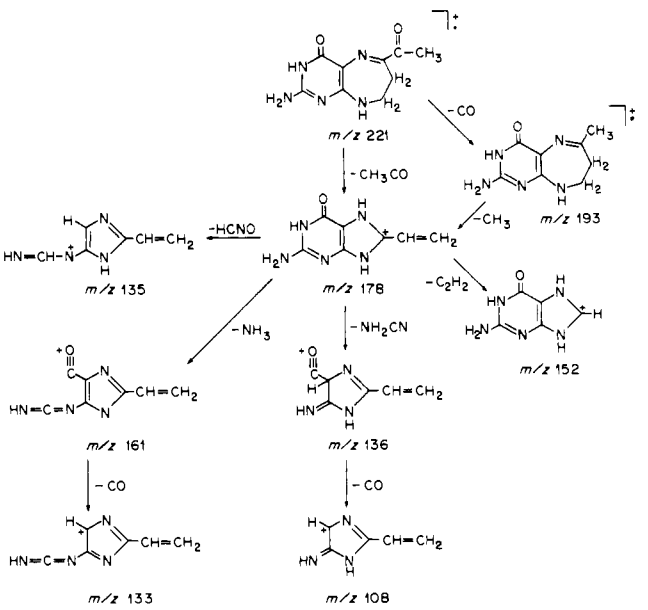

FIGURE 9: Possible ion structures in the electron ionization induced fragmentation of QS.

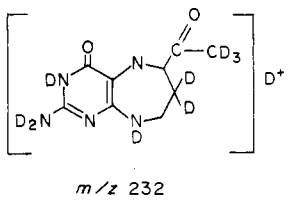

FIGURE 10: Possible sites for deuterium exchange in QS.

nine hydrogens with D_2O reagent gas, but nearly complete exchange of nine hydrogens with ND_3 to give $[^2H_9]MD^+$ = 232. The proposed structure of QS has four active N–H hydrogens and five other hydrogens that can become active (i.e., either OH or NH) through various tautomeric equilibra. The structure shown for m/z 232 (Figure 10) is proposed to account for the observed labeling pattern, in which one methylene group in the diazepine ring remains unexchanged. Structure I has an insufficient number of exchangeable hydrogens and is thus excluded on this basis as well as by the NMR and ultraviolet absorption spectra.

Gas-phase isotope exchange reactions appear to hold promise for corroboration of structural assignments. The mechanisms of these reactions are presently under investigation (Hunt & Sethi, 1980; Ausloos & Lias, 1981). The EI mass spectra of trimethylsilylated QS shows complete introduction of three Me_3Si groups (M = 437) and partial incorporation (10–15%) of a fourth Me_3Si group (M = 509). This observation is in agreement with the proposed structure (II) and is consistent with the tendency of exocyclic amino groups to incorporate only small amounts of two Me_3Si groups under the reaction conditions used (White et al., 1972).

Discussion

Several structures were considered for quench spot based on its molecular weight, as determined by mass spectrometry, and its chemical properties. All three, shown in Figure 5, seemed possible from a biosynthetic viewpoint but I was ruled out by the ultraviolet absorption and the extent of deuterium exchange as shown by mass spectrometry. The proton NMR spectrum failed to rule out either II or III, but II is the only structure consistent with the ^{13}C NMR spectrum. The evidence presented by Wiederrecht et al. (1981) consisted of the low-resolution mass spectrum, the proton NMR, and chemical properties; they proposed II as the structure. This structure is an attractive precursor for drosopterin, which Theobold & Pfleiderer (1978) showed to contain a diazepine component. The data presented herein not only confirm those of Wiederrecht et al. (1981) but offer also the ^{13}C NMR and a detailed interpretation of the high-resolution mass spectrum, both of which are necessary for establishment of structure II. To remove the ambiguity regarding the positioning of the acetyl group on position 6, 7, or 8, we have attempted to crystallize QS. Needle-shaped crystals that are too small for analysis by X-ray diffraction are easily obtained from water, but larger crystals are not yet available.

Naturally occurring diazepines are relatively uncommon. One set of imidazodiazepines are pentostatin and coformycin, which are effective as inhibitors of adenosine deaminase (Agarwal et al., 1977) and adenylic acid deaminase (Agarwal & Parks, 1977). The ring structure may be viewed as a hypoxanthine with a methylene inserted between the N-1 and C-6 positions of the purine ring to yield a homopurine. Another set consists of benzodiazepines that have various substituents attached to the rings, as summarized by Leimgruber (1973). The antibiotic anthramycin is included in this set; others are cyclopenin and cyclopenol. Both the imidazodiazepines and the benzodiazepines mentioned above are products of microbial metabolism.

Drosopterin is one of six substances identified as eye pigments in *Drosophila*. It was first considered to be a dimeric pterin structure (Pfleiderer, 1970), but, subsequently, it was shown to contain a pterin and pyrimidodiazepine in a pentacyclic ring system (Theobold & Pfleiderer, 1978). Drosopterin and isodrosopterin appeared to be enantiomers. Four other eye pigments of *Drosophila* that appear to be closely related to drosopterin are the pairs aurodrosopterins I and II and neodrosopterin and fraction e. It is possible that these also are enantiomeric pairs that contain the diazepine nucleus since their biosynthesis is stimulated by QS (Dorsett & Jacobson, 1982). Wiederrecht et al. (1981) mentioned that drosopterin synthesis is stimulated by QS (PDA is their term for this compound) but present no data on the PDA requirement and make statements regarding their unpublished observations: their heated enzyme can convert labeled dihydroneopterin triphosphate to drosopterin, isodrosopterin, and neodrosopterin but not to QS (or PDA). They also state that their enzyme preparation needs only Mg^{2+} to produce drosopterin and isodrosopterin but needs, in addition, NADH to produce aurodrosopterin. These statements are in contrast to results of Dorsett & Jcobson (1979) that drosopterin synthesis required NADH or NADPH as well as Mg^{2+}; in the absence of those cofactors, labeled QS accumulates to higher levels than in their presence. These differences may be clarified when the experimental data can be compared.

After elution from the CM-52-cellulose column QS is contaminated with sepiapterin and two minor species, one fluorescing blue and the other orange. These could all be separated from QS on the cellulose column but the stabilization of QS in ammonium chloride solutions was essential. Since several divalent cations are known to form coordinate complexes with purines (Jacobson & Turner, 1980), the effects of Ni^{2+}, Zn^{2+}, and Cd^{2+} were tested; their ability to stabilize QS decreased in the order given. They all cause a marked spectral shift when mixed with QS but little or none with sepiapterin. Sepiapterin, in contrast to QS, is stable in ammonium chloride in the absence of divalent cations. The interaction of Ni^{2+} with QS is being studied further and will be reported elsewhere. No evaluation has been made of the

effects of metal ions on the stability of QS below pH 2. Complexes of several divalent cations with another diazepine (hexahydro-1,4-diazepine-1-ethanamine) were studied spectrophotometrically (Patel & Billo, 1977).

Drosopterin obtains its name because it occurs in *Drosophila* and not in most other insects. However, drosopterins do occur in vertebrates such as amphibians (Obika & Bagnara, 1964) and the teleostean fish (Hama, 1970; Liaci, 1970). Presuming that these vertebrate drosopterins contain the diazepine-type structure, we may not be surprised to find that QS may also be present in these higher families.

Little is known about the physiological role of naturally occurring diazepines in *Drosophila*. QS and the drosopterins are major components of the pterin-related compounds in the head of this insect. The latter may be concentrated in pigment granules that line the ommatidia and provide an optical barrier between the facets of the compound eye (Clayton, 1954). It may be of interest to explore the possible physiological effects of QS since it is present in larger amounts than most of the pterins and since a diazepine with pharmacological activity is known, i.e., diazepam (or Valium). A number of benzodiazepines with pharmacological activity have been synthesized and shown to bind certain neuroreceptors (Squires & Braestrup, 1977; Möhler & Richards, 1981; Mathew et al., 1982).

The term "quench spot" will not be useful hereafter, and we propose the trivial name of 6-acetylhomopterin to replace it. It is inconvenient to employ the formal name, 2-amino-4-oxo-6-acetyl-3,4,7,8-tetrahydro-3*H*,9*H*-pyrimido[4,5-*b*]-[1,4]diazepine, and the trivial name pyrimidodiazepine does not indicate the pteridine metabolism in which this compound participates.

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