tions, if their sites overlap either of the two binding sites of the aminopyridine moieties of II.

In summary, the three fluorescent compounds described here provide sensitive probes of different functional properties of acetylcholinesterase. Because of the intrinsic sensitivity of fluorescence, the value of these probes will not be restricted to the relatively readily available eel enzyme but will be also useful in studying acetylcholinesterase from mammalian sources.

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Structure of the Light-Emitting Moiety of Aequorin†

Osamu Shimomura and Frank H. Johnson*

ABSTRACT: From 125 mg of disc electrophoretically pure aequorin extracted from *ca.* 2 tons of the jellyfish *Aequorea*, the light-emitting moiety in the bioluminescence reaction was separated as approximately 1 mg of a substance designated AF-350 (mol wt 277). The properties of AF-350, including ultraviolet and infrared absorption, nuclear magnetic resonance and mass spectra, pK_a values, and products of deuteration, acetylation, hydrogenation, and hydrolysis indicate that the structure of AF-350 is most likely 2-amino-3-benzyl-5-(*p*-hydroxyphenyl)pyrazine. This structure has further support through comparison with model compounds, namely, *Cypridina* etioluciferin and etioluciferamine. The AF-350 moiety is probably bound to the protein through the amidine part of the pyrazine ring, rather than through the phenolic hydroxyl.

The photoprotein aequorin, isolated from the bioluminescent jellyfish *Aequorea* (Shimomura *et al.*, 1962, 1963) emits visible light by an intramolecular reaction when Ca^{2+} is added. Aequorin contains a single chromophore, which is functional in the light-emitting reaction, and has been separated from the protein and designated AF-350 from its ultraviolet absorption maximum at 350 nm (Shimomura and

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Johnson, 1969, 1970). On the basis of the evidence and discussion given in the present paper, the chemical structure of AF-350 is now proposed.

Materials and Methods

Materials. AF-350 was separated and purified from pure aequorin as recently reported (Kohama *et al.*, 1971). For the present study, no more than 1 mg of AF-350 could be obtained from 125 mg of disc electrophoretically pure aequorin, which had been extracted and purified from approximately 45,000 specimens, or over 2 tons, of the jellyfish *Aequorea* by the method previously employed (Shimomura and Johnson, 1969, 1970). For purposes of comparison of properties of AF-350 with similar substances, derivatives of *Cypridina* luciferin were used. *Cypridina* etioluciferin was obtained by air oxidation of a solution of the luciferin, followed by HCl treatment. Etioluciferamine was then obtained by $Ba(OH)_2$ treatment of the etioluciferin (Kishi *et al.*, 1966a,b).

Spectrometric Measurements. Ultraviolet spectra were recorded on a Perkin-Elmer Model 402 spectrometer.

Infrared spectra were measured under three different conditions, namely, (1) 500 μg of AF-350 dissolved in 0.4 ml of chloroform on a Perkin-Elmer Model 421 spectrophotometer using a 0.5-mm cell, (2) 400 μg of AF-350 on a NaCl plate evaporated from ether solution on the same instrument as before, and (3) 16 μg of AF-350 in a micro KBr pellet on a Digilab Model FTS-14 infrared interferometer at the Sadtler Research Laboratories, Philadelphia. In the first two methods, the sample was recovered by washing the cell and the NaCl plate with ether.

The nuclear magnetic resonance (nmr) spectrum of AF-350 (500 μg) dissolved in $CDCl_3$ (0.4 ml) was obtained on a Varian Model HA-100, C-1024 by time averaging of 25 scans at the Physical Chemistry Department of the Hoffmann-LaRoche Inc., Research Laboratories, Nutley, N. J.

Mass spectra were obtained by the Hitachi-Perkin-Elmer Model RMU-6D of the Morgan-Schaffer Corp., Montreal, Canada. High-resolution mass spectrometry was carried out at the Physical Chemistry Department of Hoffmann-LaRoche Inc., Nutley, N. J.

pK_a Measurement. pK_a values were determined spectrophotometrically using a Perkin-Elmer Model 402 spectrophotometer at X5 sensitivity and a Corning Model 12 pH meter equipped with a semimicro combination electrode. AF-350 (8 μg) in 10^{-3} M NH_4Cl (2.4 ml) containing 33 or 66% ethanol was titrated with 1 N NaOH or 1 N HCl at 25°. The lower pK_a was determined at 340–350 nm, and the higher pK_a at 300–310 nm.

Deuterium Exchange. Dried AF-350 (70 μg) was dissolved in a mixture of 2 drops each of CD_3OD (99%) and D_2O (99.9%), then the solution was left standing for 24 hr at room temperature with a tight stopper. After evaporating the solvent under vacuum, the sample was redissolved in 1 drop of CD_3OD for convenience of handling this sample for mass spectrometry.

Acetylation. AF-350 (50 μg) was heated with acetic anhydride (0.1 ml) and pyridine (0.1 ml) in a sealed-glass tube at 100° for 1.5 hr. After evaporation under a vacuum, the residue was dissolved in 1 drop of ethanol.

Catalytic Hydrogenation. AF-350 (30–150 μg) in 5 ml of ethanol containing acetic acid (5 drops, 2 drops, 10 μl, or none) was hydrogenated with 0.3 mg of PtO_2 catalyst at room temperature for a period of between 1 and 20 hr. Six experiments were carried out with various amounts of AF-350 and acetic acid for various reaction times. After removing the catalyst by centrifugation, the solvent was evaporated under a vacuum.

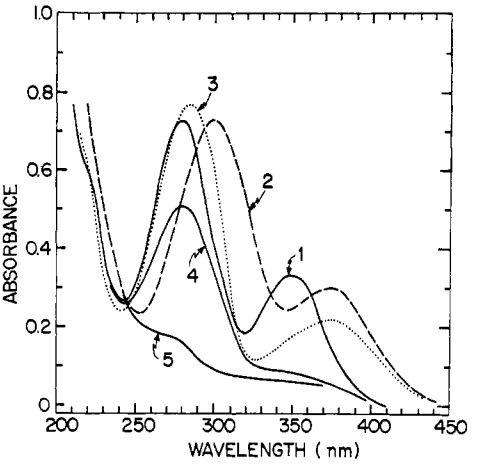


FIGURE 1: Ultraviolet spectra of AF-350 in 50% ethanol (1), in 50% ethanolic 0.01 N NaOH (2), in 50% ethanolic 0.1 N HCl (3), alkaline $K_3Fe(CN)_6$ oxidation product in ethanol (4), and product of hydrogenation (20 hr in the presence of 2 drops of acetic acid) in ethanol (5). The amount of sample, *ca.* 10 μg/ml for 1, 2, and 3, and also approximately equivalent concentration as AF-350 for 4 and 5. Light path, 1 cm. The absorbance of 1.0 corresponds to approximately ϵ 27,000.

Alkaline $K_3Fe(CN)_6$ Oxidation. One drop of 0.1 M $K_3Fe(CN)_6$ was added to AF-350 (100 μg) dissolved in 0.1 ml of 0.1 N NaOH which was prepared with 50% ethanol. After 10 min the mixture was acidified by addition of a small drop of acetic acid, then evaporated to approximately half-volume, and finally the product was extracted with ether from the residual solution.

Hydrolysis. AF-350 (16 μg) or *Cypridina* etioluciferin (160 μg) was heated with 6 N HCl (0.2 ml) in evacuated, sealed tubes at 115° for two different periods, namely, 20 and 70 hr. HCl was evaporated in a vacuum desiccator containing P_2O_5 and KOH, and the residue was analyzed by a Beckman amino acid analyzer. For the hydrolysate of AF-350, analysis for basic amino acids was omitted.

Results

General Properties of AF-350. AF-350, obtained as an almost colorless crystalline powder, was found to be soluble in alcoholic solvents, ether, water, and slightly soluble in chloroform. The melting point was not measured; however, the compound could be distilled (or sublimed) under a high vacuum at 150–200°. Heating 5 μg of this compound with *p*-dimethylaminobenzaldehyde, carried out as a spot test on filter paper (Feigl, 1960), resulted in a strong yellow color, suggesting the presence of an aromatic amino group in AF-350. Treatments of AF-350 with $NaBH_4$ at room temperature, or with 1 N NaOH at 100° for 30 min did not affect the structure as judged from the mass spectra of the products.

Spectrometric Properties. The ultraviolet absorption maximum of AF-350 at 350 nm at neutral pH showed bathochromic shifts by addition of acid as well as of alkali (Figure 1). Solutions of AF-350 in alcohols or ether were strongly blue fluorescent (λ_{max}^{EtOH} 433 nm).

The infrared spectrum measured in chloroform solution was generally poor, due to an insufficient concentration of the sample, although some clear absorption bands, including a

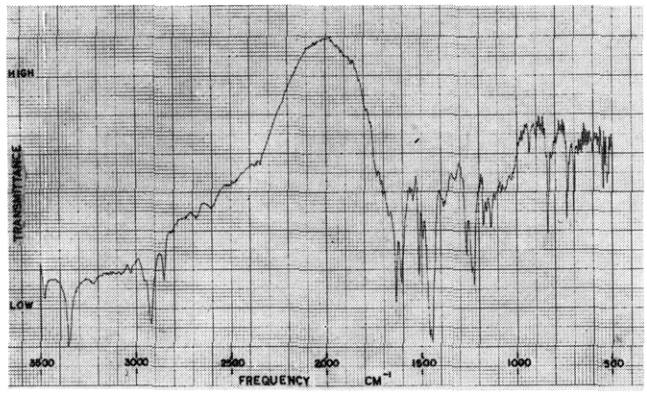


FIGURE 2: Infrared spectrum of 16 μg of AF-350 obtained by the micro KBr pellet method. Transmittances at 3200 and 2000 cm^{-1} were 37 and 76%, respectively.

TABLE I: *Partial Result of High-Resolution Mass Spectrometry.*

Measured	Fragments Lost from M^+	Calcd[a]
277.1202	(M^+)	277.1215
276.1162	H	276.1137
262.1019	CH_3	262.0980
261.1043	NH_2	261.1028
250.1105	CHN	250.1106
249.1035	CH_2N	249.1028
234.0929	CH_3N_2	234.0919
233.0841	CH_4N_2	233.0841
222.0919	$C_2H_3N_2$	222.0919

[a] As $M^+ = C_{17}H_{15}N_3O^+$.

sharp peak at 3610 cm^{-1}, were observed. The infrared spectrum measured on a NaCl plate was essentially the same as the spectrum measured by a micro KBr pellet method (Figure 2), although the former suffered from a somewhat greater scattering and a lower resolution. Absorption bands at 1450 and 2920 cm^{-1} were considered to be mostly due to impurities, probably hydrocarbon or grease-like substances, because the structure of AF-350 proposed in the present study would not produce such signals.

The nmr spectrum (Figure 3) (δ values given in parts per million) showed one pair of characteristic doublets at around 7–8. Signals between 0.7 and 2.0 were considered to be due to impurities, the same as in the infrared spectrum. With the nmr spectrum, however, small amounts of additional impurities, contributed in detectable traces from the $CDCl_3$ solvent, and possibly also from using the same sample in two preceding measurements of the infrared spectrum, might have been involved.

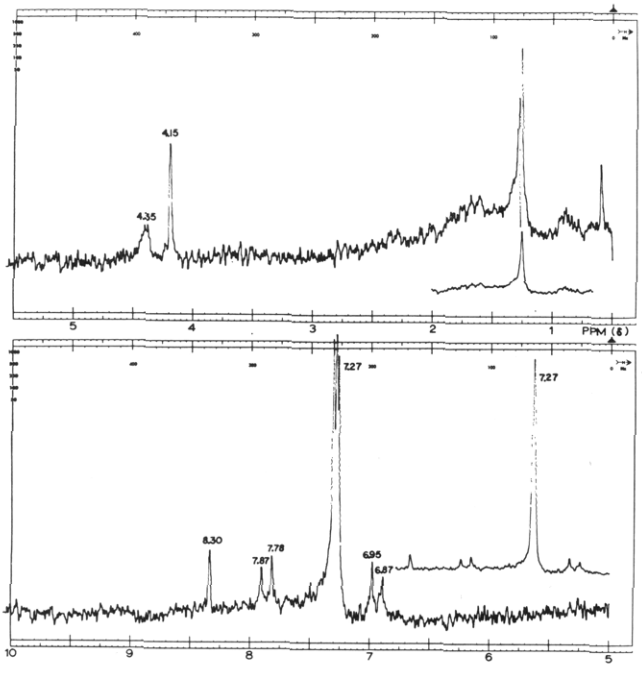


FIGURE 3: 100-MHz nuclear magnetic resonance spectrum of 500 μg of AF-350 in 0.4 ml of $CDCl_3$, after time averaging 25 scans. Partial scans for the peaks of 1.25 and 7.27 ppm at a reduced sensitivity are also included.

The mass spectrum of AF-350 (Figure 4A) indicated, in addition to the molecular weight of 277, an aromatic nature of the compound by the outstanding abundance of the molecular ion as well as by the fairly intense peak of *m/e* 77. High-resolution mass measurement (Table I) revealed characteristic expulsions of CH_3, NH_2, HCN, and H_2CN from the molecular ion.

pK_a of AF-350. pK_a values obtained by the ultraviolet absorption method were as follows: in 33% ethanol, 2.3 and 10.1; in 66% ethanol, 2.3 and 10.7. The lower pK_a values were considered to be less precise than the higher ones due to fluctuation of up to ± 0.15 pK unit. In connection with these pK_a values, adsorption behavior of AF-350 on ion exchangers was examined. Thus, AF-350 was found to be adsorbed on DEAE-cellulose (free base form) and subsequently eluted with a pH 7.0 buffer, suggesting that an acidic group, having a pK_a value higher than 7 is present. Moreover, AF-350 was found to be adsorbed on CM-cellulose (H^+ form) and subsequently eluted with 40% ethanol, suggesting the presence of a weak basic group which has a pK_a value close to that of the carboxyl group in CM-cellulose. From the data described it seems evident that the higher pK_a, *ca.* 10, should be attributed to an acidic group, and the lower pK_a to a basic group.

Deuteration and Acetylation. Treatment of AF-350 with D_2O, followed by mass spectrometry, revealed the presence of three exchangeable hydrogens in one molecule of the compound by the appearance of *m/e* 280 (Figure 4B). The incompleteness of the deuteration could be at least partially due to the reversal of deuteration caused by the exposure of the small amount of product (70 μg) momentarily to atmospheric humidity.

Mass spectrometry of the acetylation product indicated the presence of three acetyl groups, although the content of the triacetyl compound seemed considerably smaller than that of the diacetyl compound, as seen from the following data measured at 70 eV: *m/e* 276 (relative intensity 46), 277 (100), 278 (21), 301 (20), 302 (6), 318 (8), 319 (72; monoacetyl), 320 (17), 343 (5), 361 (78; diacetyl), 362 (18), 403 (8; triacetyl), 404 (2).

Hydrogenation. The results of catalytic hydrogenation analyzed by means of mass spectrometry were extremely complicated. No molecular species produced by a simple hydrogenation was detected in the product whereas molecular

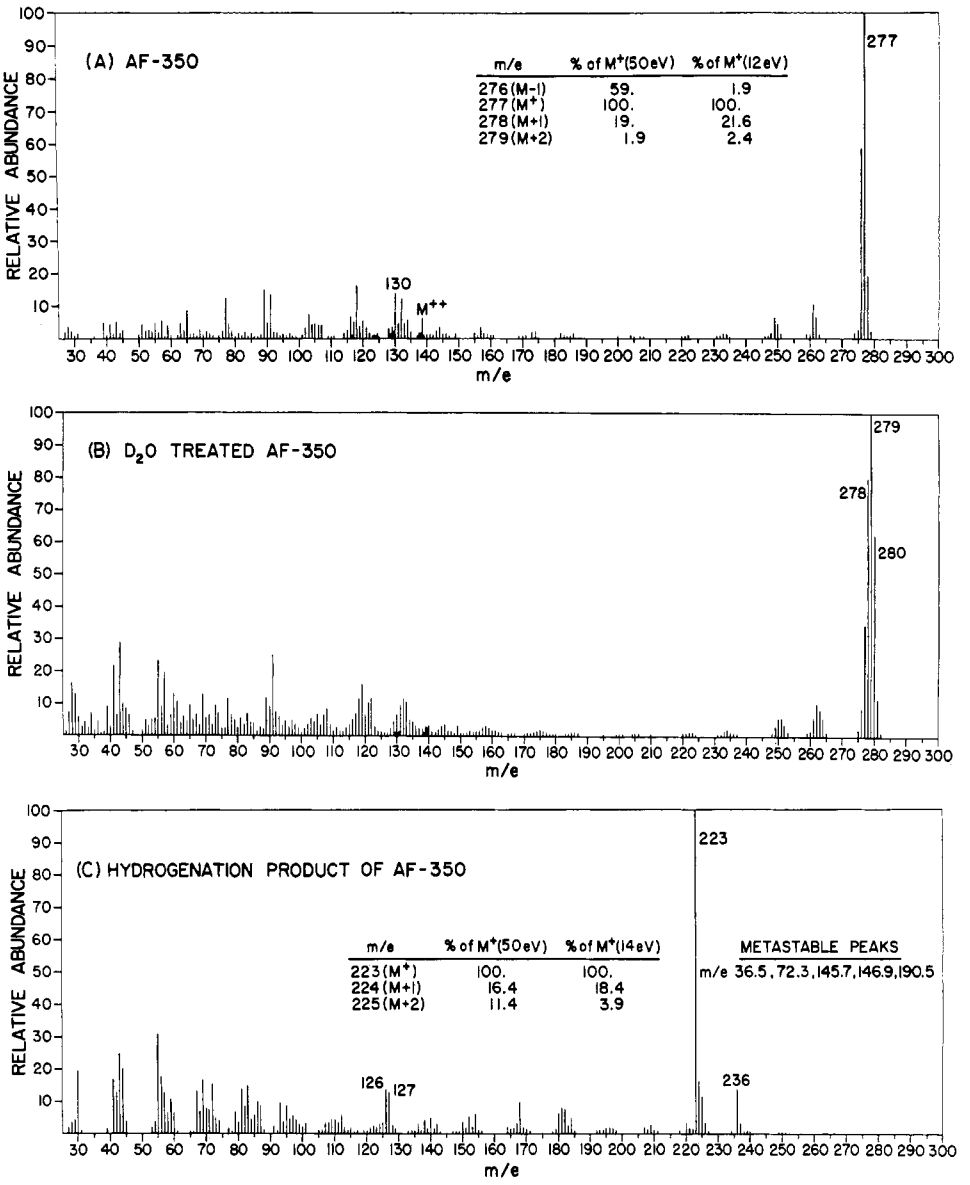


FIGURE 4: Mass spectra of AF-350 (A), after deuteration (B), product of hydrogenation (20 hr; see text) (C), at 50 eV.

species with mol wt 209, 219, 220, 223, 225, 230, 231, and 236 were consistently found in various proportions, indicating that the reductions were always accompanied by hydrogenolyses. Sometimes several more minor species (*m/e* 205, 207, 211, etc.) and uncertain species (*m/e* 177 and 182) were found. Often the most prominent component in the earlier stage of reduction was mol wt 219 species, whereas in the later stage of the reduction mol wt 223 species was most prominent. The mass spectrum of the mol wt 223 species, which was obtained by separating it as much as possible from the other components in the mass spectrometer, is shown in Figure 4C. The metastable peak at *m/e* 72.3 (223 → 127 + 96) seems to be important in elucidating the structure of mol wt 223 component. The ultraviolet spectrum of the product at the later stage of the reduction was, as expected, featureless (Figure 1). The product at the earlier stage of reduction, however, indicated the presence of a phenol group (Figure 5).

Hydrolysis. Heating of AF-350 in 6 N HCl at 115° for 20 hr afforded 0.13 mole of glycine and 0.028 mole of phenylalanine. No other acidic or neutral amino acid was detected in the product. Extension of the heating time to 70 hr did not change the yields of the amino acids.

Discussion

Molecular Formula of AF-350. The result of high-resolution mass measurement of the molecular ion, namely, 277.1202 (Table I), allowed four kinds of possible formulas, namely, $C_{11}H_{19}NO_7$ (277.1162, calcd M + 1 = 12.85%), $C_{12}H_{15}N_5O_3$ (277.1175, calcd M + 1 = 15.23%), $C_{17}H_{15}N_3O$ (277.1215, calcd M + 1 = 19.79%), and $C_{10}H_{19}N_3O_6$ (277.1274, calcd M + 1 = 12.49%). After consideration of the actually measured value of M + 1, 21.6% (Figure 4A), $C_{17}H_{15}N_3O$ was chosen as the molecular formula of AF-350. This formula has the total number of rings plus double bonds of 12, indicating an aromatic nature of the compound.

Presence of p-Hydroxyphenyl Group. The presence of phenolic OH was indicated by infrared absorption bands at 3610 (in chloroform), 3360, and 1230 cm^{-1} (in KBr pellet), and by pK_a of an acidic group at about 10. Also the ultra-

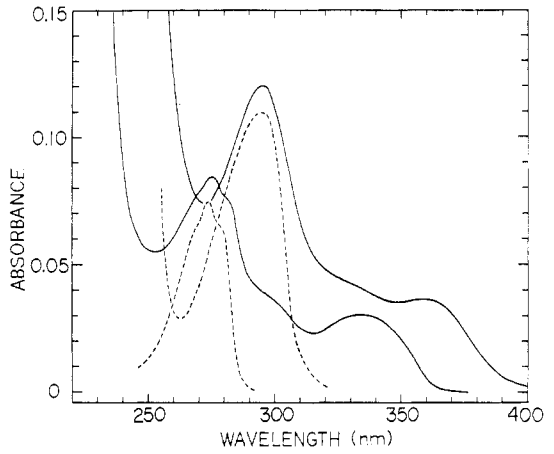


FIGURE 5: Ultraviolet spectra of the hydrogenation product of AF-350 (solid lines) and phenol (broken lines), in 0.5 ml of 90% ethanol (λ_{max} 275 nm for both) and after addition (to each) of 10 μl of 10% NaOH (λ_{max} 295 nm for both). AF-350 (150 μg) had been hydrogenated for 3 hr in the presence of PtO_2 (0.3 mg) and acetic acid (2 drops) in ethanol (5 ml). The concentration of the hydrogenation product was approximately equivalent to 10 μg of AF-350/ml, and the concentration of phenol was *ca.* 3.4 μg/ml. Thus, the absorbance of 0.1 corresponds roughly to ϵ 2700.

violet spectra of the catalytic hydrogenation product, at an early stage of the reduction indicated the presence of a phenol chromophore in the product, as shown in Figure 5. The presence of *p*-disubstituted benzene was indicated in the nmr spectrum as a pair of doublets namely, 6.91 (J = 8 Hz) and 7.82 (J = 9 Hz), and also by an infrared signal at 840 cm^{-1}.

Because a second benzene ring was found in AF-350 as a benzyl group (*vide infra*), whereas the molecular formula of AF-350 does not allow the presence of more than two benzene rings in one molecule, the phenolic OH must be bound to the *p*-substituted benzene. Thus, the presence of a *p*-hydroxyphenyl group was concluded. Furthermore, the spectral characteristics in the ultraviolet (Figure 1) as well as the nmr doublet at 7.82 favor the view that the *p*-hydroxyphenyl group is conjugated with another chromophore, possibly with another aromatic ring.

Benzyl Group and Amino Group. In the nmr spectrum of AF-350 (Figure 3), only five aromatic protons were apparent, namely, as the signals of a para-disubstituted benzene (4 H) and an unidentified singlet at 8.3 (1 H), whereas the molecular formula of AF-350 suggested the presence of more than five aromatic protons. In the circumstances that the signals of some other aromatic protons were coincidentally hidden under the strong, sharp singlet at 7.27 which was due to an impurity of $CHCl_3$ in the solvent, presence of a monosubstituted benzene, namely, a phenyl group, which often gives a sharp singlet at 7.2–7.3 when attached to a carbon atom, would be highly probable.

Although the high-resolution mass measurements (Table I) indicated that the fragment *m/e* 262 of AF-350 should be $M^+ - CH_3$, the nmr signal of the CH_3 group, which would be expected to be on a nitrogen, double-bonded carbon or aromatic skeleton, could not be found. Furthermore, comparison between the mass spectrum of AF-350 and that of deuterated AF-350 (Figure 4B) revealed that, in deuterated AF-350, a loss of CDH_2, instead of CH_3, from the molecular ion had occurred. Thus, AF-350 should contain a CH_2 group, but not a CH_3 group, and furthermore the *m/e* 262 fragment should be ($M^+ - CH_2$ and H).

The presence of the NH_2 group, which had been indicated by the positive color reaction with *p*-dimethylaminobenzaldehyde, was further supported in the high-resolution mass spectrometry by a fragment of ($M^+ - NH_2$).

Subtraction of the four groups discussed above, namely, *p*-hydroxyphenyl, phenyl, CH_2, and NH_2, from the molecular formula of AF-350 leaves a formula of C_4HN_2 which should have the rings plus double bonds of 4. The hydrogen remaining here must be the aromatic proton which is responsible for the nmr signal of 8.3. Efforts to arrange C_4HN_2 to a five-, four-, or three-membered ring, followed by rebinding of the above-mentioned four groups, were not successful; no structure could be found which was compatible both with the nmr singlet of 8.3 and with the pK_a value of a basic group at *ca.* 2.3.

When the C_4HN_2 is arranged as a six-membered heteroaromatic ring (ring plus double bonds = 4), the four groups named above must bind to the three carbons of the ring by single bonds. The *p*-hydroxyphenyl group must then conjugate with the other chromophore as discussed above, and the NH_2 group must be directly attached to the heteroaromatic ring because a CH_2NH_2 structure would certainly give a much higher pK_a value than 2.3; therefore, among the four groups, the phenyl group must be connected through a methylene bridge to the heteroaromatic ring. It follows that a benzyl group is present. The methylene group in such a benzyl structure seems to be consistent with the nmr signal of AF-350 at 4.15 (2 H, singlet); *e.g.*, 2-benzylpyridine, 4.18 (Varian Associates, 1962, 1963). The presence of the benzyl group was further supported by the mass spectrum (*m/e* 91), by infrared absorption bands (740 and 700 cm^{-1}), and also by the formation of phenylalanine by hydrolysis.

Structure of AF-350. The foregoing discussion suggests that AF-350 has a structure of a six-membered heteroaromatic ring containing two ring-nitrogen atoms, to which one amino group, one *p*-hydroxyphenyl group and one benzyl group are substituted respectively at three different ring-carbon atoms.

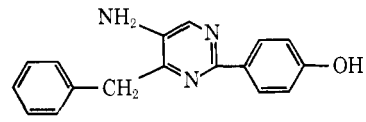
The possibilities that AF-350 may include a skeleton of 3-aminopyridazine, 4-aminopyridazine, or 4-aminopyrimidine were ruled out, because the pK_a values of these amino heteroaromatic rings, namely, 5.2, 6.7, and 5.7, respectively (in water, Albert, 1968), were too high, even taking into account the effects of the other two substituents, in comparison with the basic pK_a of 2.3 of AF-350 (in 33% ethanol).

2-Aminopyrimidine (pK_a = 3.5; Albert, 1968) as the skeleton of AF-350 was judged to be still too basic from the pK_a values of model compounds: *e.g.*, 2-amino-4,6-diphenylpyrimidine, pK_a = 3.78 (Brown and Harper, 1963), 2-amino-4,6-dimethylpyrimidine, pK_a = 4.99 (Brown *et al.*, 1966). Additionally, the skeleton seemed highly unlikely to produce glycine and phenylalanine by hydrolysis. Thus, we ruled out the possibility that AF-350 may contain a 2-aminopyrimidine skeleton.

The pK_a of 5-aminopyrimidine (2.6 in water; Albert, 1968) is a fairly favorable value, and substitutions of this ring allow three possible structures, among which 4-(*p*-hydroxyphenyl)-5-amino-6-benzylpyrimidine and 2-benzyl-4-(*p*-hydroxyphenyl)-5-aminopyrimidine could be ruled out due to lack of a skeleton of glycine or phenylalanine, thus leaving 2-(*p*-hydroxyphenyl)-4-benzyl-5-aminopyrimidine (I) as the only possible structure in this group.

2-Aminopyrazine has a pK_a of 3.1 in water (Albert, 1968). Considering the influence of substituents and the difference in solvents, this pK_a of 3.1 seems in quite satisfactory agreement with the pK_a of 2.3 of AF-350, even though the nmr

chemical shift for any of three protons on the ring is 8.1 or less (Brown *et al.*, 1968; Cox and Bothner-By, 1968). Six different structures can be written by substituting the ring with a *p*-hydroxyphenyl group and a benzyl group. However, the nmr signal of AF-350 is 8.3 rather than the 8.1 of 2-aminopyrazine. It is to be expected that a *p*-hydroxyphenyl substituent will cause such a downfield shift to a proton when the proton is bound in the ortho position. Consequently, positions 5 and 6 are the only possible sites for binding the substituent and the proton. Thus, only two kinds of structures would become possible; namely, 2-amino-3-benzyl-6-(*p*-hydroxyphenyl)pyrazine (II) and 2-amino-3-benzyl-5-(*p*-hydroxyphenyl)pyrazine (III).



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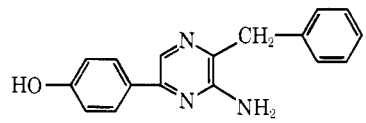

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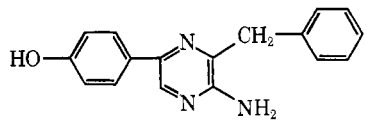

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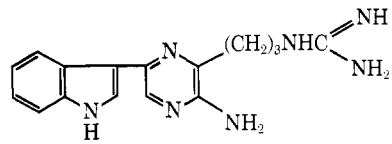

IV

The foregoing discussion leads to three possible structures, namely I, II, and III, for AF-350. To decide between these possibilities, efforts were made to weigh their likelihood according to certain characteristics as follows.

Although no data were found in the literature on the nmr spectra of 5-aminopyrimidine or of its derivatives with adequate substituent(s), the heteroaromatic proton of structure I would probably give a δ value of about 8.0, due to the strong upfield shift caused by an amino group at the ortho position (proton at position 3 of pyrimidine, 8.6; Jackman and Sternhell, 1969). On the other hand, for II and III, δ values of 8.2–8.5 would be expected (*e.g.*, 2-amino-5-phenylpyrazine, 8.5 in Me_2SO-d_6; Sugiura *et al.*, 1969; also, see below for etioluciferin). The pK_a value of the basic group of I, which would be expected to be approximately 0.5 unit lower than that of II or III, could be slightly below the pK_a value of the basic group of AF-350.

Repeated mass spectral measurements of AF-350 always showed a weak but consistent fragment ion at *m/e* 222 (Figure 4A). This fragment, which was identified to be ($M^+ - C_2H_3N_2$) by high resolution mass measurement (Table I), would be expected from structures I or III, but not from II. The formation of glycine and phenylalanine in acid hydrolysis is understandable far more easily according to structures II or III than I; also the relatively higher yield for glycine seems to favor structure III rather than II.

None of the data discussed above indicate any inconsistency between AF-350 and structure III, whereas discrepancies are evident with respects to structures I and II.

Structure III has the same 2-amino-3,5-disubstituted pyrazine as *Cypridina* etioluciferin (IV) (Kishi *et al.*, 1966a,b). Thus it is interesting and perhaps useful to compare the properties of this convenient model compound, IV, to those of AF-350 in an effort to adduce further evidence bearing on structure III.

The nmr signal of the pyrazine proton in the structure IV at 8.3 (in $(CD_3)_2SO$), and pK_a values of IV and etioluciferamine (NH_2 instead of guanidyl in IV), respectively, at 2.9 and 2.4 (in 50% ethanol) were reported by Kishi *et al.* (1966b) in quite good agreement with the values found in the present study of AF-350. Changes in the ultraviolet spectra of AF-350 at pH 1.5–3.5 resemble those of etioluciferin or etioluciferamine, although some differences are evident as expected by the differences of an indolyl and a *p*-hydroxyphenyl group. Hydrolysis of IV in 6 N HCl at 115° for 20 hr or 70 hr afforded 0.055 mole of glycine and 0.01 mole of arginine. Considering the difference of substituents, this result seems consistent with the results of hydrolysis of AF-350.

In regard to the mass spectrum of AF-350, fragments found between *m/e* 115 and 200 are, if AF-350 has structure III, generally in accordance with the fragmentation scheme of the pyrazine skeleton of etioluciferamine (Kishi *et al.*, 1966a,c). For example, elimination of $C_6H_5CH_2CN$ from III, followed by loss of NH_2CN or (H + HCN), gives *m/e* 118 or 132, respectively. Elimination of HOC_6H_4CN and H from III gives *m/e* 157, which further loses HCN resulting in *m/e* 130.

The close similarity in properties of AF-350 and IV indicate that they correspond in having a skeleton of 2-amino-3,5-disubstituted pyrazine. On this basis, together with the evidence discussed in previous paragraphs, structure III is proposed as the most likely structure of AF-350.

The nature of the skeleton is of special interest in itself. Compounds having an aminopyrazine skeleton are rarely encountered among natural products; in fact, they are thus far known or believed to occur only among bioluminescence systems. Thus, in addition to the foregoing evidence that such a ring occurs in AF-350 derived from the luminescence system of *Aequorea*, it has been established that it occurs also in the luciferin substrate of the light-emitting system of the ostracod crustacean *Cypridina hilgendorfii* (Kishi *et al.*, 1966a,b). Furthermore, it appears possible that an almost identical pyrazine skeleton occurs in the luciferin of the sea pansy, *Renilla* (DeLuca *et al.*, 1971).

The Structure of AF-350 Moiety in Aequorin. Because of similarities in the ultraviolet spectrum (in the 350 nm region) between AF-350 and BFP (blue fluorescent protein, the product of the luminescence reaction of aequorin with Ca^{2+}) as shown by Shimomura and Johnson (1969), it seems likely that, in BFP, the AF-350 moiety is bound to the protein part with no significant change of the chromophore. Structure III suggests two likely binding sites of AF-350 to the protein part, namely, the phenolic OH and the amidine part of the pyrazine ring. The former, however, was found to be not bound because the 335-nm peak in the spectrum of BFP at neutral pH is shifted to 365 nm at alkaline pH with an approximate pK_a of 9.5, thus leaving the latter as the more likely possibility.

In regard to the structure of the chromophore in native aequorin, a distinct difference between the ultraviolet spectrum of native aequorin (Shimomura and Johnson, 1969) and that of AF-350 indicates that the AF-350 moiety in native aequorin

must be in a substantially different chromophoric state. A small adsorption peak of aequorin at 470 nm (ϵ roughly 1000) which disappears after the bioluminescence reaction might indicate the presence of a quinoid structure. The value of ϵ seems too low to indicate a fused heterocyclic ring such as is known to occur in *Cypridina* luciferin. A more detailed study of this important point is currently in progress at this laboratory.

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Added in Proof

The proposed structure (III) is strongly supported by the properties of the two model compounds, A and B, which have been recently synthesized by Dr. Y. Kishi at Nagoya University, Japan: (A) 2-amino-5-(*p*-hydroxyphenyl)pyrazine, uv max (50% C_2H_5OH) 278 and 350 nm, (50% C_2H_5OH–NaOH) 299 and 372 nm, (50% C_2H_5OH–HCl) 281 and 370 nm; nmr (Me_2SO-d_6) 6.88 (d, 2 H, J = Hz), 7.61 (d, 2 H, J = 9 Hz), 8.00 (s, 1 H), 8.22 (s, 1 H); (B) 2-amino-3-benzyl-5-phenylpyrazine, nmr ($CDCl_3$) 4.20 (s, 2 H), 7.23 (s, 5 H), 7.42 (2 H), 7.96 (2 H), 8.38 (s, 1 H).

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