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THE STRUCTURE AND SYNTHESIS OF ANNULOLINE, AN OXAZOLE ALKALOID OCCURRING IN ANNUAL RYE GRASS*

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Abstract—Annuloline, the fluorescent alkaloid which occurs in the roots of *Lolium multiflorum* (annual rye grass) has been shown by synthesis to have the structure 2-(*trans*-3,4-dimethoxystyryl)-5-(4-methoxyphenyl) oxazole. This appears to be the first instance in which an oxazole ring has been demonstrated in a naturally occurring compound. The isomer, 5-(3,4 dimethoxyphenyl)-2-(*trans*-4-methoxystyryl) oxazole has also been synthesized.

INTRODUCTION

TWO SPECIES of rye grass are commonly grown in the United States, *Lolium multiflorum*, an annual, and *Lolium perenne*, a perennial. Because of their similar morphology, the seeds are difficult to distinguish by inspection. However, upon germination, the two varieties can be readily differentiated from one another owing to the brilliant bluish-white fluorescence exhibited by the roots of the annual under long u.v. light. The perennial roots do not normally fluoresce. This difference is utilized by seed analysts to distinguish between the seeds of the two species.[1] The characteristic of fluorescence is reported to be inherited as a Mendelian dominant.[2,3]

Previous work in this laboratory resulted in the isolation and purification of the fluorescent material.[4] The compound given the trivial name, annuloline, was shown to have the formula $C_{17}H_{10}NO(OCH_3)_3$. It is a weakly basic phenylpropanoid alkaloid. The present report establishes the structure of annuloline and describes its synthesis. A preliminary note concerning these studies has appeared elsewhere.[5] Annuloline appears to be the first oxazole whose natural occurrence has been reported.

RESULTS

The value for the molecular weight of annuloline as determined by the Rast method (in camphor) was found to be 356 indicating that the empirical formula weight, 337, was indeed the true molecular weight.

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[1] U.S. Department of Agriculture, Testing Agricultural and Vegetable Seeds, p. 103, Government Printing Office, Washington, D.C. (1952).

[2] L. CORKHILL, *Nature*, 130, 134 (1932).

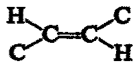
[3] P. A. LINEHAM and S. P. MERCER, *Nature* 131, 202 (1933).

[4] B. AXELROD and J. B. BELZILE, *J. Org. Chem.* 23, 919 (1958).

[5] R. S. KARIMOTO, B. AXELROD, J. WOLINSKY and E. D. SCHALL, *Tetrahedron Letters* 3, 83 (1962).

The presence of three methoxyl groups was verified by the N.M.R. spectrum which indicated a ratio of nine methoxyl hydrogens (at 3·77 ppm) to ten aromatic and/or olefinic hydrogens (at 6·48–7·56 ppm). Overlapping precluded more specific assignments in the latter region. Methyl, methylene and tertiary carbon hydrogens were definitely excluded.

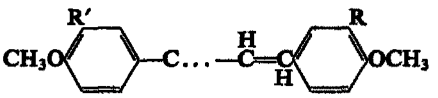
The i.r. spectra, showing no OH, —NH or C═O, suggested that the fourth oxygen atom might be in an ether structure, and that the N might be in a tertiary amine. A pronounced band at 10·35 μ indicated a trans-ethylenic group,



This band was lost on hydrogenation. In further confirmation of this grouping the N.M.R. spectrum of the hydrogenation product showed a signal at 3·0 ppm characteristic of $—CH_2—Ar$.

Significant data were obtained from oxidation experiments. Thus permanganate oxidation yielded anisic acid and veratric acid. The nature of the two acids was established by potentiometric determination of their acid equivalence value, by mixed melting point determinations and by the identity of the i.r. and u.v. spectra with those of the corresponding authentic compounds. Chromate oxidation gave a good yield of anisic acid. Since the sum total of veratric and anisic acids exceeded one equivalent per mole of annuloline it was assumed that these acids did not arise from common carbon atoms.

The identification of the two aromatic acids among the oxidation products and the strong presumption that one of the aryl groups was part of a styryl moiety (in view of the u.v. spectrum, as well as the i.r. and N.M.R. studies discussed above) permitted the structural assignment (I). Inasmuch as $C_{20}H_{19}NO_4$ corresponded to 12 rings plus double bond, the remaining atoms (2C, H, O and N) required a grouping which provided an additional 3 rings plus double bonds. Since the uptake of H_2 in the presence of PtO_2 was 10 moles per mole of annuloline a cyclic enolic ether containing N was proposed to meet these requirements.



R′ = H or OCH_3; R = OCH_3 or H, resp.

(I)

In considering the possibility of a heterocyclic ring, containing two double bonds, a weakly basic nitrogen and an enolic ether, the fluorescent properties of the 2,5 disubstituted oxazoles (2,5-diphenyloxazole is a well-known phosphor used in scintillation detection of radioisotopes) commended the oxazole ring to our attention. Several reasons made this speculation attractive.

(1) A spot of 2,5-diphenyloxazole placed on filter paper fluoresced blue–white in long u.v. light, but turned green after exposure to fumes of HCl. Treatment with fumes of NH_3 resulted in the restoration of the original fluorescence. This was precisely the behaviour of annuloline.

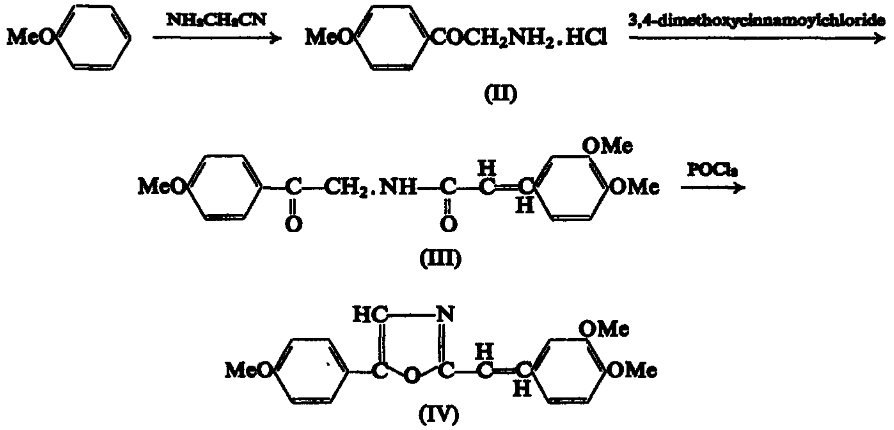
(2) The u.v. spectra of annuloline resembled that of various 2,5 aryl disubstituted oxazoles. For example, the absorption maximum and extinction coefficient found for annuloline[4] were

350 mμ (log $\epsilon = 4 \cdot 56$) while the corresponding values[6] for 5-phenyl-2-styryl oxazole were 334 mμ (log $\epsilon = 3 \cdot 19$). Methoxylation could conceivably account for the bathochromic shift.

(3) It has been previously observed that the addition of small amounts of oxygen-containing solvents to a solution of annuloline in petroleum ether strongly depressed fluorescence.[4] Similar behaviour was observed with 2,5-diphenyloxazole.

(4) Cornforth[7] had reported that in the hydrogenation of diphenyl oxazoles hydrogenolysis of the cyclic ether linkage occurred in addition to the reduction of the aromatic rings.

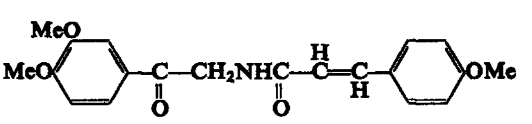
In order to assign the styryl and phenyl moieties to their positions on the oxazole rings, the syntheses of the two isomeric oxazoles were undertaken. The synthetic pathway leading to 2-(3,4-dimethoxystyryl)-5-(4-methoxyphenyl) oxazole (IV) is shown in Scheme 1.



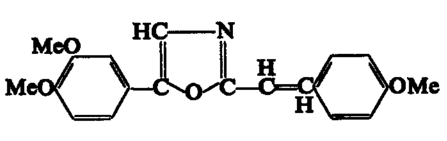
SCHEME 1

The product, IV, proved to be identical with natural annuloline on the basis of mixed m.p. determination, u.v. spectrum, i.r. spectrum, fluorescence activation and emission spectra and elementary analysis.

The isomer, VI, was synthesized in an analogous manner, starting with 1,2-dimethoxybenzene and going through the isomeric amide (V).



(V)



(VI)

The product, VI, differed from annuloline in having a slightly different i.r. spectrum, and it gave a depressed melting point on being mixed with authentic annuloline.

The close resemblance of annuloline to 2,5-diphenyloxazole in structure and its marked fluorescent qualities suggested its possible use as a fluor. It was accordingly tested as a replacement for 2,5-diphenyloxazole in a mixture normally used for counting ^{14}C in a Packard

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[6] D. O. OTT, F. N. HAYES, E. HANSBURY and V. N. KERR, *J. Am. Chem. Soc.* 79, 5448 (1957).

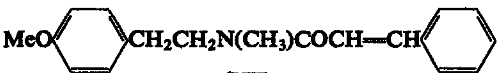
[7] J. W. CORNFORTH in *Heterocyclic Compounds*, Ed. R. C. ELDERFIELD, Vol. 5, p. 327, J. Wiley and Sons, Inc., New York (1957).

Tri-Carb Counter. The counting rate obtained when diphenyloxazole was replaced with an equal weight of annuloline was 34% of the normal rate.

DISCUSSION

Annuloline is apparently the first naturally occurring oxazole to be identified. Katritzky and Lagowski[8] have stated in their recent monograph on heterocycles that oxazoles are not found in Nature. The absence of references to oxazole alkaloids in the literature contributed to the difficulty in arriving at the structure of annuloline since oxazoles were not considered until experimental evidence forced them into focus as a strong possibility.[9]

Now that the structure of annuloline is known, it appears feasible to undertake the study of its biogenesis. The relationship of annuloline to phenylpropanoid precursors is obvious. The synthetic pathway itself involving an N-cinnamyl-phenylethylamine suggests what is at least superficially a reasonable biological route from two common "building blocks" encountered in plants, phenylethylamine and cinnamic acid. It is of interest to note that an amide, N-(2-*p*-anisylethyl)-N-methyl cinnamide (VII), similar to that proposed has actually been isolated from the bark of the southern prickly ash.[10]



(VII)

It is hoped that a comparative study of the individual steps will reveal the enzymic basis for the difference between the fluorescent and non-fluorescent species of rye grass.

EXPERIMENTAL

Isolation of Annuloline

The procedure by which annuloline was obtained from rye grass was essentially similar to that described previously.[4] In a typical preparation 5000 g of seeds of *Lolium multiflorum* was spread uniformly over 20 sheets of filter paper (Whatman No. 1, 46 cm × 57 cm) and kept moist for 12 days. On the fourteenth day the aerial protions of the seedlings were clipped off and discarded. The filter paper in which the roots were matted was reduced to pieces about 10 × 10 cm in size and extracted with about 36 l. of petroleum ether. Two days, during which the mixture was stirred occasionally, were allowed for extraction. Since the germinations took place at room temperatures, there was considerable variation in the yields of extractable fluorescence. An average yield was about 1×10^8 fluorescent units. Fluorescence was measured on dilutions made in petroleum ether using a Coleman fluorophotometer (Model 12C) fitted with B and PC filters. The reference standard used was quinine sulfate solution (0·3 μg/ml in 0·1 N H_2SO_4), with which the instrument was set to read 75.

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[8] A. R. Katritzky and J. M. Lagowski, *Heterocyclic Chemistry*, p. 206, Methuen and Co. Ltd., London (1960).

[9] Indeed, a number of other possibilities were considered, including substituted quinolines isomeric with annuloline. Inasmuch as the structure of annuloline was ultimately established these compounds are not of especial interest here. However, it is noted here that a hitherto undescribed quinoline was prepared by one of us (R. S. K.): 2-(3,4-dimethoxystyryl)-4-chloro-7-methoxyquinoline. R. S. Karimoto, *The Structure and Synthesis of Annuloline, a Naturally Occurring Oxazole Alkaloid*, Ph.D. Thesis, Purdue University (1962).

[10] F. B. Laforge and W. F. Barthel, *J. Org. Chem.* 9, 250 (1944).

The combined extracts were concentrated to 540 ml and stored at −10° to −20° for several weeks. A waxy precipitate which formed was discarded. In a typical isolation the concentrates from five such runs were combined and further concentrated to 280 ml containing $3\cdot64 \times 10^8$ fluorescence units. The hydrochloride of annuloline was precipitated by passing gaseous HCl into the extract. After cooling to 0° the mixture was centrifuged and the supernatant discarded. The resultant black tarry residue was dissolved in a minimum volume of ethanol (125 ml) and treated with 20 ml of a 10% (w/v) ethanolic solution of picric acid. Recrystallization of the picrate (0·41 g) from ethanol yielded yellow needles (0·27 g, m.p. 223–225°, decomp.). The mother liquor yielded an additional 0·08 g (m.p. 210–219°, decomp.). The combined products were augmented with 0·08 g from a previous run, suspended in benzene and shaken with 1 N NaOH until no further picric acid was extracted. The benzene solution was washed twice with equal volumes of water. Upon evaporation of the benzene 0·21 g (m.p. 106–109°) of annuloline was obtained. A total of 2·4 g of annuloline was obtained from the seedlings produced from 340 kg of rye grass seed.

Since the elemental analysis of the above preparation was not in satisfactory agreement with the earlier values,[4] it was further purified. In a typical procedure, 0·37 g of the above material was dissolved in 11 ml of benzene–petroleum ether (1:1 v/v). HCl was passed through the cooled solution and the annuloline hydrochloride was recovered by centrifugation. The precipitate was washed by heating it briefly with 1·5 ml ethanol on a steam bath. The residue was mixed with 5 ml H_2O containing several drops of 2 N NH_4OH and extracted with benzene (5 ml + 3 times with 1 ml). The combined benzene extract was washed with three 1·0 ml portions of H_2O and evaporated to dryness to give 0·21 g of light yellow crystals (m.p. 110–114°). Recrystallization from ethanol yielded 0·13 g of annuloline (m.p. 112–114°), having an analysis in complete agreement with that reported originally.[4]

Formation of anisic and veratric acids from annuloline. Annuloline (46·7 mg) was added to alkaline $KMnO_4$ (172 mg $KMnO_4$, 5 ml H_2O + 4 drops 6 N NaOH). After 30 min refluxing, ethanol was added to reduce excess $KMnO_4$. The insoluble MnO_2 was removed by filtration and the filtrate acidified with H_2SO_4. Upon evaporation of the ether extract, a yellow oily residue was obtained. Washing the residue with a small amount of ether removed the oil and left a white solid, which was recrystallized from ether–acetone to give 5·1 mg of a substance melting at 180–182°. Paper chromatography of the unrecrystallized products from a previous oxidation in *n*-butanol-NH_4OH-$(NH_4)_2CO_3$[11] showed two substances when viewed under short u.v. light (253·7 mμ). Both substances were acidic as revealed by a methyl red indicator spray. One spot fluoresced blue, while the other appeared as a dark, quenching area. The recrystallized substance from the present experiment gave only the blue fluorescent spot. A micro-potentiometric titration of the crystalline material gave a value of 182 for the neutral equivalent (theory for dimethoxy benzoic acid, 182). Veratric acid and the substance in question showed identical behaviour on chromatography ($R_f = 0\cdot20$, blue fluorescence in short u.v.). The u.v. spectra of the two substances were similar: the unknown had maxima at 283 mμ ($\log \epsilon = 3\cdot77$), 260 mμ ($\log \epsilon = 3\cdot97$) and 217 mμ ($\log \epsilon = 4\cdot10$). The corresponding values obtained with authentic veratric acid were: 281 mμ ($\log \epsilon = 3\cdot68$), 260 mμ ($\log \epsilon = 3\cdot97$) and 217 mμ ($\log \epsilon = 4\cdot29$). The i.r. spectra of the two substances in KBr pellets were identical.

In a subsequent oxidation of a larger amount of annuloline (100 mg) by the above procedure 30 mg of veratric acid was isolated. The filtrate after the removal of the veratric acid was extracted with ether. Evaporation of the ether left a yellow solid which was extracted with 5% $NaHCO_3$. After filtration to remove an insoluble orange residue, the $NaHCO_3$ extract

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[11] M. E. Fewster and D. A. Hall, *Nature*, **168**, 78 (1951).

was acidified, whereupon 5·2 mg of a light brown precipitate was obtained (m.p. 175°). On paper chromatography the substance gave a dark quenching spot corresponding to that previously noted. Anisic acid and the unknown showed dark quenching spots on paper chromatography with an identical R_f (0·26). The unknown acid had a single absorption maximum at 254 mμ (log $\epsilon = 4{\cdot}22$), compared to 253 mμ (log $\epsilon = 4{\cdot}15$) for anisic acid. The i.r. spectra of the two substances in KBr pellets were identical. Oxidation of annuloline with chromic acid yielded a preponderance of anisic acid, whose identity was established by i.r. spectroscopy and mixed melting point determinations.

Hydrogenation of annuloline. A solution of annuloline, 12 mg, in ethanol, was stirred overnight in an atmosphere of H_2 in the presence of PtO_2. The catalyst and solvent were removed and the residue crystallized from a small amount of ethanol to give colorless clusters of crystals, m.p. 96–97°; λ_{max}, 279 mμ, $\epsilon = 4{\cdot}36$.

Synthesis of 5-(3,4-Dimethoxyphenyl)-2-(4 Methoxystyryl) Oxazole

4-*Methoxycinnamic acid.* *p*-Hydroxycinnamic acid was methylated to give *p*-methoxycinnamic acid methyl ester.[12] The product was deesterified by hydrolysis with NaOH to give the desired compound (m.p. 172–174°).

2-*Amino*-3′,4′ *dimethoxyacetophenone hydrochloride.* Following the method of Moed *et al.*,[13] 15 g of veratrole and 9·4 g aminoacetonitrile hydrochloride was treated with 30 g of anhydrous $AlCl_3$ in 60 g of dry nitrobenzene to yield 19 g of the crude detone. The product was recrystallized from benzene (m.p. 220–221°, with decomposition).

N-3,4-*Dimethoxyphenacyl*-4-*methoxycinnamamide* (*V*). The amide was prepared by a modification of the procedure of Hayes *et al.*[14] The 4-methoxycinnamyl chloride (prepared from 1·85 g of 4-methoxycinnamic acid and thionyl chloride) was treated with 2·3 g of the above aminoketone hydrochloride in 30 ml of dry pyridine to give 2·8 g of a crude brown product. The material was recrystallized from benzene as light brown needles. These were recrystallized twice from acetone–ethanol (1:3, v/v), the second time in the presence of activated charcoal. The final yield of *N*-3,4-*dimethoxyacetophenone*-4-*methoxycinnamamide* from 2·3 g of crude material was 0·73 g; m.p. 170–173°. (Found: C, 67·80; H, 6·03; N, 4·03. $C_{20}H_{21}NO_5$ required: C, 67·61; H, 5·91; N, 3·94%.)

5-(3,4-*Dimethoxyphenyl*)-2-(4-*methoxystyryl*) *oxazole* (*VI*). The cyclization of the above ketoamide (V) to the corresponding oxazole was carried out by an adaptation of the procedure of Hayes *et al.*[14] The dark brown tarry product obtained from 1·75 g of the ketoamide in phosphorus oxychloride (70 ml) was collected by filtration and partitioned between benzene and 2 N NaOH. Evaporation of the benzene fraction produced tan-colored needles contaminated with a reddish-brown tar. The tar was removed by washing with a minimal amount of benzene. Recrystallization from benzene followed by two crystallizations from ethanol gave 0·23 g of the 5-(3,4-*demethoxyphenyl*)-2-(4-*methoxystyryl*) *oxazole* (m.p. 110–111°). (Found: C, 71·18; H, 5·63; N, 4·24. $C_{20}H_{19}NO_4$ required: C, 71·22; H, 5·64; N, 4·15%.) The m.p. was slightly lower than that of natural annuloline (114°) and was depressed when mixed with annuloline.

The u.v. spectrum in cyclohexane showed maxima similar to that of annuloline (350 mμ). However, the intensity found at 275 mμ with the synthetic compound was greater than that

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[12] W. WILL, *Ber.* **20**, 294 (1887).

[13] H. D. MOED, M. ASSCHER, P. J. A. VAN DRAAHEN and H. NIEWAND, *Rec. Trav. Chim.* **71**, 933 (1952).

[14] F. N. HAYES, B. S. ROGERS and D. O. OTT, *J. Am. Chem. Soc.* **77**, 1850 (1955).

observed for annuloline and the band at 350–360 mμ was considerably sharper. The activation and fluorescence spectra of the two substances were remarkably similar.

Synthesis of 2-(3,4-*Dimethoxystyryl*)-5-(4-*Methoxyphenyl*) *Oxazole* (*Annuloline, IV*)

2-*Amino*-4′-*Methoxyacetophenone hydrochloride* (*II*). This aminoketone was synthesized in a manner analogous to that described above for the 3,4-dimethoxy homolog. The yield of crude product from 9·8 g of amino acetonitrile hydrochloride and 12 g of anisole was 12·7 g. The product, after recrystallization from ethanol, melted at 203–204° with decomposition.

N-4-*Methoxyphenacyl*-3,4-*dimethoxycinnamamide* (*III*). This substance was synthesized in a manner similar to that described above for the isomeric amide. 2 g of 3,4-dimethoxycinnamic acid were converted to the acyl chloride and reacted with 1·85 g of 2-amino-4′-methoxyacetophenone hydrochloride. The crude *N*-4-*methoxyphenacyl*-3,4-*dimethoxycinnamamide* (1·9 g) was recrystallized from benzene and acetone–ethanol; yield 0·36 g, m.p. 189–191°. (Found: C, 67·58; H, 5·92; N, 3·97; OCH_3, 26·69. $C_{17}H_{12}NO_2\,(OCH_3)_3$ required: C, 67·61; H, 5·91; N, 3·94; OCH_3, 26·2%.)

2-(3,4-*dimethoxystyryl*)-5-(4-*methoxyphenyl*) *oxazole* (*IV*). Using the $POCl_3$ treatment described above, *N*-4-*Methoxyphenyl*-3,4-*dimethoxycinnamamide* (2·05 g) was converted to 2-(3,4-dimethoxystyryl)-5-(4-*methoxyphenyl*) *oxazole*, which was isolated as the picrate. The picrate was suspended in benzene and shaken with 1 N NaOH to liberate the alkaloid. The product after recrystallization from benzene weighed 1·69 g. After two recrystallizations from ethanol, the material (0·23 g) melted at 114–114·5° (Found: C, 71·30, 71·33; H, 5·62, 5·62; N, 4·25, 4·26; OCH_3, 27·6. $C_{17}H_{10}NO(OCH_3)_3$ required: C, 71·22; H, 5·64; N, 4·15; OCH_3, 27·6%.) The u.v. and i.r. spectra of this compound as well as the activation and emission fluorescence spectra were identical with the corresponding spectra of annuloline isolated from rye grass. Moderate to strong absorption peaks were seen at 6·22, 6·70, 6·92, 8·02, 9·76, 10·29, and 13·0 μ. The fluorescence value for the synthetic compound was 656 units/μg per ml compared to 660 units for natural annuloline.

ANALYSES

Elemental analyses, methoxyl determinations, quantitative hydrogenations and molecular weight determinations were performed by Huffman Microanalytical Laboratories, Wheatland, Colorada, and Geller Microanalytical Laboratories, Bardonia, New York. Methoxyl determinations were also carried out in this laboratory by the Zeisel method.[15]

Infra-red spectra were obtained, using KBr pellets as well as CCl_4 solutions, in a Baird i.r. spectrophotometer. Ultra-violet spectra were obtained in a Cary Recording Spectrophotometer. The N.M.R. spectra were made in a Varian HR60 Nuclear Magnetic Resonance Spectrometer. The determinations were made at 60 mc in a carbon tetrachloride solution using tetramethylsilane as a standard.

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[15] J. B. NIEDERL and V. NIEDERL, *Organic Quantitative Microanalyses*, p. 187, J. Wiley and Sons, Inc., New York (1938).