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Chemistry

A valid model for the mechanism of oxidation of tryptophan to formylkynurenine—25 years later

(dye-sensitized photooxygenation/labile hydroperoxy tryptophan metabolites/x-ray analysis)

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ABSTRACT **The dye-sensitized photooxygenation of DL-tryptophan in aqueous solution leads to the tricyclic compound 2-carboxy-3a-hydroperoxy-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-*b*]indole which, on reduction with dimethyl sulfide, furnishes two diastereoisomeric alcohols separable by fractional crystallization into a higher melting (mp 254°–256°) and a lower melting (mp 228°) diastereoisomer. Each of these alcohols was correlated with one of the analogous pair of isomeric 1,2-dicarbomethoxy analogs by alkaline hydrolysis and by x-ray analysis. In this way, the 3a-hydroxy-1,2-dimethoxycarbonyl-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-*b*]indole, mp 163°–164°, was shown to have the *trans* configuration with regard to the relative positions of the hydroxyl and carbomethoxy groups and that, on alkaline hydrolysis, it produced the isomer with mp 228°, which therefore also has the *trans* configuration. The mechanism of the smooth thermal rearrangement of the (presumably ring-chain tautomeric) tryptophan hydroperoxy intermediates to formylkynurenine is discussed with its implications for the biological oxidation by tryptophan 2,3-dioxygenase.**

The first direct chemical conversion of tryptophan (**1**) to formylkynurenine (**4**) was by ozonolysis (1). The biochemical degradation of L-tryptophan by L-tryptophan oxygenase, a dioxygenase [L-tryptophan:oxygen 2,3-oxidoreductase (decyclizing), EC 1.13.11.11], served as one of the first demonstrations of the incorporation of both ^{18}O moieties of molecular oxygen ($^{18}O_2$) (2) and prompted many model studies involving intermediary hydroperoxides arising from indole substrates through catalytic oxidation (3) or photooxygenation (4, 5).

(*See structure cut on top of next page.*)

Earlier studies (6–13; for reviews, see refs. 14 and 15) on the sensitized photooxygenation of **1** led to complicated mixtures of products in which kynurenine was detected (7) and formylkynurenine was isolated in low yield (12). In accord with the general mechanism of oxidations of enamines (16), of which indoles are a special case (3), the 3-hydroperoxyindolenine **2** (17) was postulated to be the primary intermediate capable of rearranging to formylkynurenine (**4**), whether *via* an energetically unfavorable dioxetane **3** (18) or *via* hydrated intermediates **5** and/or **6** (19, 20) may be left open at this point. Our recent studies on the dye-sensitized oxygenation of *N*(b)-methoxycarbonyltryptamine and -DL-tryptophan methyl ester [**9**; *N*(b) is the basic nitrogen, and *N*(a) is the indolic one] have provided unambiguous evidence for the intermediary formation of a 3-hydroperoxyindolenine (4, 21–24). Furthermore, we have found a new reaction pathway to kynurenine *via* the long-sought-after 3a-hydroperoxyhexahydropyrroloindole (23, 24).

The reinvestigation of the dye-sensitized photooxygenation of tryptophan itself in aqueous solution has now led to the major product, 3a-hydroperoxypyrrolidinoindole (**7**) which easily rearranges to formylkynurenine (**4**) upon warming.

EXPERIMENTAL

When an aqueous solution (300 ml) of DL-tryptophan (**1**) (1 g, 5 mmol) containing EtOH (15 ml) and Rose Bengal (0.4 mmol) was photooxygenated at 0°–5° for 2.5 hr, followed by reduction with dimethyl sulfide, the 3a-hydroxyhexahydropyrroloindole (**8**) was obtained as a mixture of two diastereoisomers that were readily separated by fractional crystallization from water to give **8a**, mp 254°–256° (28%) and **8b**, mp 228° (28%), in addition to 23% of recovered tryptophan (**1**). The spectral properties of **8** were in complete accord with those reported by Savige (25) who obtained **8** by oxidation of **1** with peracetic acid. Neither formylkynurenine nor *N*(b)-formylkynurenine have so far been detected (thin-layer chromatography; UV absorption) in the reaction mixture.

On the other hand, DL-formylkynurenine (**4**) was isolated in yields up to 26% from the reaction mixture [in addition to **8** (11%)] when heated for 30 min at 100° immediately after the irradiation until the starch–KI test became negative. These results are clearly expected on the basis of analogous experiments in which the sensitized photooxygenation of **9**, followed by silica gel column chromatography, provided **11**, **12**, and **13** (23). The tricyclic hydroperoxide **10** has now been isolated by sensitized photooxygenation of **9** in 40% yield as a mixture of *cis* and *trans* isomers with respect to the hydroxy and ester groups. The properties of **10**, a mixture of diastereoisomers, are λ_{max} (EtOH) 242, 303 nm; nuclear magnetic resonance (NMR) ($CDCl_3$, δ 2.40–2.80 (m, 2H, CH_2), 3.23, 3.64, and 3.73 (s, 6H, CO_2Me), 4.40 (m, 1H, $CHCO_2Me$), 4.60–5.40 (broad, 1H, OOH or NH, exchangeable), 5.60 (finely split s, 0.5H, NCHN), 5.80 (finely split s, 0.5H, NCHN), 6.50–6.90, 7.00–7.30 (m, 4H, aromatic H), 9.40 (broad s, 1H, NH or OOH, exchangeable); mass 308 (20)M^+, 292(60)M − O, 291(8) M − OH, 290(43) M − H_2O, 281(18), 233(48), 231(20), 203(34), 199(17), 146(59), 145(59), 132(100), 130(46). The hydroperoxide **10** was rearranged to **11**, **12**, and **13** by silica gel in CH_2Cl_2; reduction with Me_2S quantitatively gave **11** as a mixture of *cis* and *trans* isomers in a 1:1 ratio readily separable by column chromatogra-

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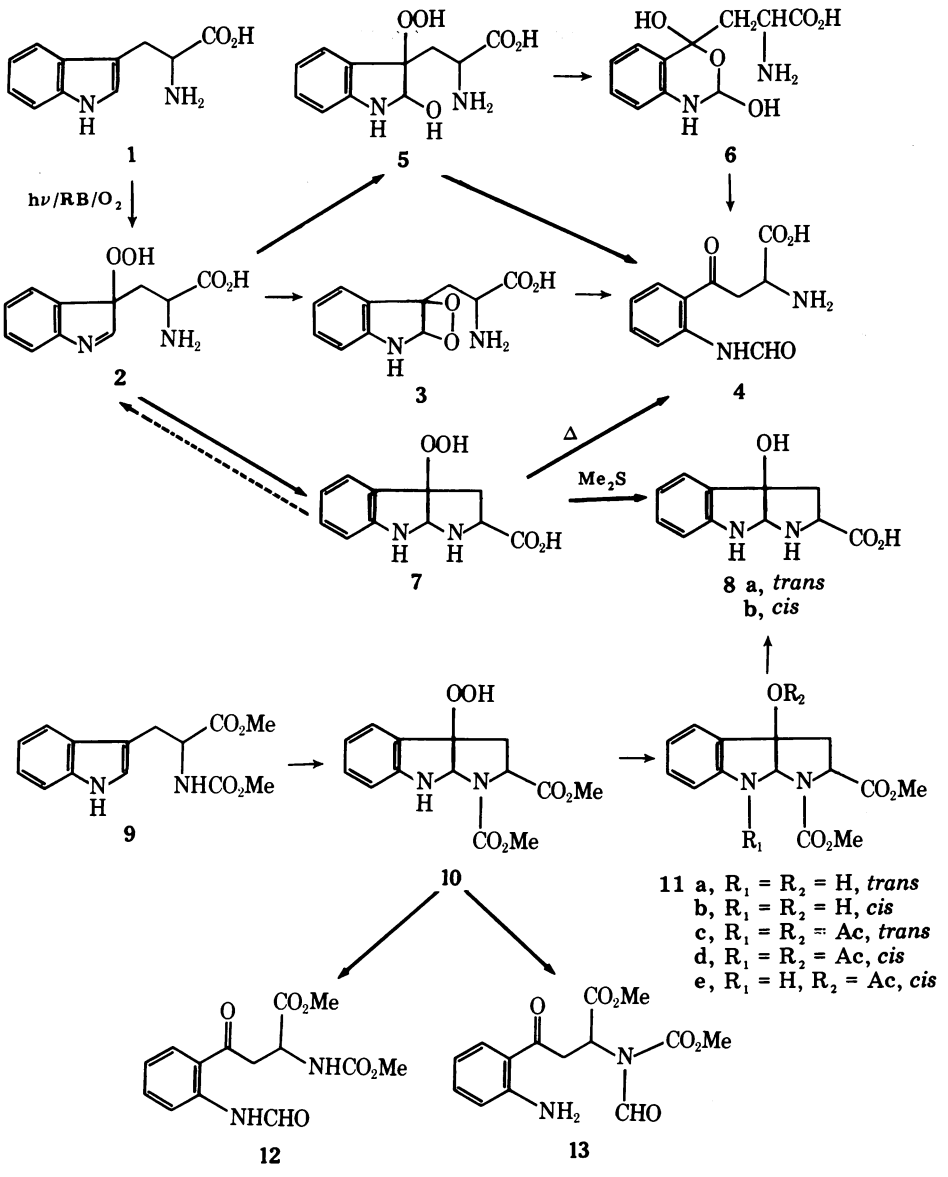
Abbreviation: NMR, nuclear magnetic resonance.



phy: The *trans*-isomer (*vide infra*) **11a** showed mp 163°–164°,§ λ_{max} (EtOH) nm(ϵ) 243(8140), 302(2370); NMR ($CDCl_3$) δ 2.30–3.00 (m, 2H, CH_2), 3.18, 3.21 (s, 3H, CO_2Me), 3.65, 3.78 (s, 3H, NCO_2Me), 4.55 (m, 1H, $CHCO_2Me$), 5.17 (s, 1H, NCHN), 6.50–6.90, 7.00–7.50 (m, 4H, aromatic H); ν_{max} (KBr) cm^{-1} 3340 (NH, OH), 1740, 1695(CO); mass 292(10) M^+, 274(3) M − H_2O, 233(13), 146(34), 132(100). The *cis* isomer (*vide infra*) **11b** showed mp 124°–125°; λ_{max} (EtOH) nm(ϵ) 240.5(8150), 296(2490); NMR($CDCl_3$) δ 2.50(m, 2H, CH_2), 3.06, 3.36 (s, 1H, NH or OH, exchangeable), 3.65, 3.78 (s, 6H, CO_2Me), 4.38 (q, 1H, $CHCO_2Me$), 4.80, 5.15 (s, 1H, OH or NH exchangeable), 5.44 (s, 1H, NCHN); ν_{max} (KBr) cm^{-1} 3460 (NH, OH), 1750, 1705 (CO); mass 292(27)M^+, 274(6), 132(100).

The stereochemistry of **11a** was unequivocally determined to be *trans* by x-ray analysis of a single crystal which belonged to the monoclinic space group, $P2_1/c$ with a = 10.331(6)Å, b = 11.898(6)Å, c = 15.148(8)Å, and β = 131.7(2)°. There are four molecules per unit cell, corresponding to a calculated crystal density of 1.40 g/cm^3. The structure was solved by the symbolic addition procedure for centrosymmetric crystals (26) and the results are displayed in Fig. 1.¶

Alkaline hydrolysis of the *trans* isomer **11a** gave the lower melting isomer **8a** in 88% yield, in addition to trace amounts of **8b** detected on thin-layer chromatography, whereas the *cis* isomer **11b** gave **8b** as a single product in 84% yield.‖ Accordingly, **11b** and **8b** have *cis* stereochemistry, and **11a** and **8a** are *trans* isomers. Treatment of *trans* isomer **11a** at room temperature with acetic anhydride in pyridine gave only a diacetate **11c**, mp 162.5°–163.5° in 99% yield. By contrast, **11b** gave both a diacetate **11d**, mp 150°–151° (77%) and a monoacetate **11e**, mp 121°–122.5° (21%).

§ All new compounds isolated and characterized by melting points rendered correct analyses for C, H, and N. The analogous oxygenation of L-tryptophan derivatives likewise gave two isomers of **11** that were readily separated by chromatography but have failed to crystallize so far.

¶ Complete crystallographic data will be published elsewhere by J. Flippen. For the computer-assisted preparation of the stereodiagram, see C. K. Johnson, ORTEP, ORNL-3794, Oak Ridge National Laboratory, Oak Ridge, TN (1965).

‖ The diastereoisomers **8a** and **8b** obtained by alkaline hydrolysis of **11a** and **11b**, respectively, were identical (infrared, UV, NMR, mass, thin-layer chromatography) with the compound obtained by direct oxygenation and reduction of **1**.

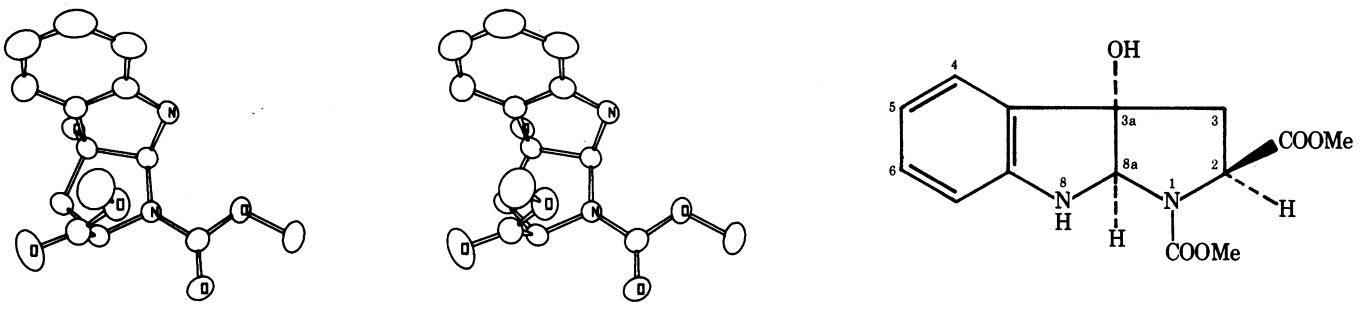
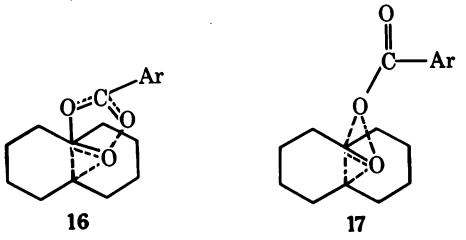


FIG. 1. Stereodiagram of 1,2β-Dimethoxycarbonyl-3aα-hydroxy-1,2,3aα,8,8aα-hexahydropyrrolo[2,3-b]indole (**11a:** arbitrarily chosen optical antipode corresponding to L-tryptophan).

DISCUSSION

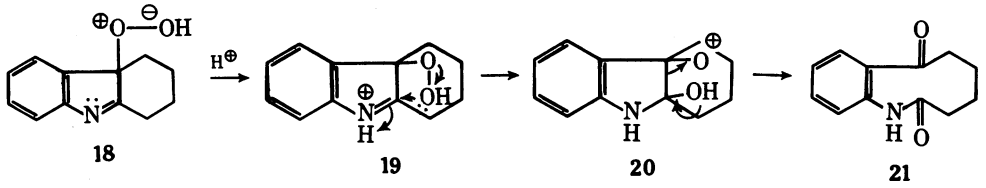
The ethylamino (alanine) side chain in the 3-hydroxyperoxy intermediate **2**, both in organic solvents as well as in water, undergoes easy intramolecular addition to form the 3a-hydroperoxyhexahydropyrroloindoles which rearrange to formylkynurenine (**4**) on warming. This facile transformation, **2** → **7** → **4**, no longer necessitates consideration of the hydrated intermediates **5** or **6** which were suggested previously in the sensitized photooxygenation of tryptophan. Although details of the mechanism for this transformation remain to be elaborated, formylkynurenine (**4**) presumably does not arise from tryptophan (**1**) *via* the energetically unfavorable intermediate dioxetane (18), which, in a formal sense, represents the extreme of a transition state with the process of bond breakage and formation being concerted as in many reactions involving cationoid rearrangements of hydroperoxides. Thus, ^{18}O studies of the heterolytic cleavage and *intramolecular* rearrangement of *trans*-9-decalylhydroperoxide benzoate to 1,6-epoxycyclodecyl benzoate and 6-hydroxycyclodecanone exclude the 5-membered (**16**) in favor of the 4-membered transition state (**17**) (27)



16 **17**

in which the dotted lines represent bonds in the process of forming or breaking. The symbolism in such representations points to the slow evolution of our understanding of the nature of bonding forces in transition states in general, in regard to which the late S. Winstein has made an important contribution to the "anchimeric acceleration of peroxide bond heterolysis" (28).

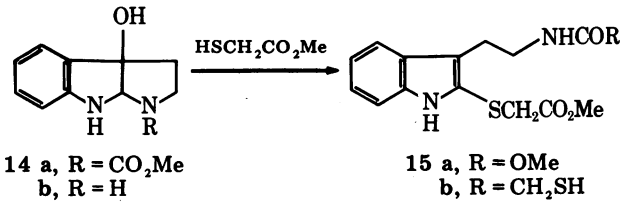
In an analogous fashion, the strong effect of acid catalysis on the rearrangement of 11-hydroperoxytetrahydrocarbazolenine (**18**) to 1-aza-8,9-benzocyclonona-2,7-dione (**21**) has been interpreted as a heterolytic cleavage of the peroxide bond with concerted intramolecular addition of the anionic oxygen to the strongly electron-attracting protonated azomethine group (**19**)



18 **19** **20** **21**

involving a *quasi* four-membered transition state (**20**) with concomitant breakage and formation of oxygen bonds (3, 29). The transition state in the rearrangement of the hydroperoxide of octahydroquinoline (30) is related and should involve neither a real interceptable dioxetane nor a peroxirane (31). Whether the thermal rearrangement of the hydroperoxides **7** (or **2**) proceeds by homolytic or heterolytic mechanisms (or both) still has to be established.

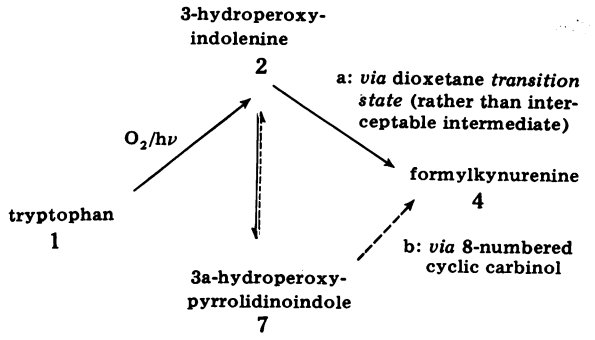
A dioxetane intermediate **3** could arise only if **7** were in equilibrium with the 3-hydroperoxyindole **2**. Such a ring-chain tautomerism **7** ⇌ **2** is probably negligible at room temperature and in the absence of acid. It requires strong nucleophiles to displace *N*(b) (directly or through a rearrangement) and open up the hexahydropyrroloindole **7**. When **14a** was heated with methyl thioglycolate (32) or **14b** was left for 24 hr with methyl thioglycolate in CH_2Cl_2 in the presence of silica gel, the 2-sulfur-substituted indoles **15a**** and **15b**, respectively, were formed in high yield (33). The conditions of the thermal rearrangement of **7** → **4** could be strong enough to open up **7** to **2** which should rearrange spontaneously



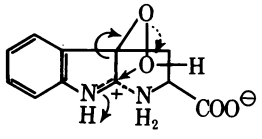
14 a, R = CO_2Me
b, R = H

15 a, R = OMe
b, R = CH_2SH

** The characteristics of **15a** are: chromatographically homogeneous oil; λ_{max} (EtOH) nm 224, 285, 293, 301[¶,‖]; ν_{max} (KBr) cm^{-1} 3350 (NH), 1735, 1720, 1700 (CO), 1530 (CONH), 1280 (C—O); NMR ($CDCl_3$) δ 3.10 (t, 2H, CH_2), 3.50 (m, 2H, CH_2N), 3.69, 3.77 (s, 6H, CO_2Me), 4.84 (broad s, 1H, NHCO), 9107 (s, 1H, NH); mass 322 (37) M^+, 234 (100) M − CH_2NHCO_2Me, 202 (23), 174 (31). For **15b:** λ_{max} (EtOH) nm 225, 285, 292, 301; ν_{max} (KBr) cm^{-1} 3350, 3250 (NH), 2550 (SH), 1730 (CO_2Me), 1650 (NHCO), 1535 (NHCO), 1295 (C—O); mass 338 (21) M^+, 234 (73), 202 (32), 174 (100).



to formylkynurenine (**4**). A plausible transition state **22** is reminiscent of the ring-chain tautomerism observed with the "ozonides" of indoles (34). If **7**



22

were to rearrange directly, an 8-membered unstable cyclic carbinol should result capable of furnishing *N*(a)- or *N*(b)-formylkynurenines. These are observed under different conditions of decomposition (4).

Summary. The present experiments establish and characterize intermediates and a rational pathway of relevance to the biological oxidation of tryptophan (**1**) to formylkynurenine (**4**). The validity of this model extends thus far only to nonenzymatic oxidation. It is unlikely that the hydroperoxyindolenine **7** will be accepted by, or function with tryptophan oxygenase as a partner in the oxidation-reduction sequence that involves an obligatory ternary tryptophan–2,3-dioxygenase–O_2 complex. As a potential transition state analog the *cis* or *trans* form of **7** might interfere with the enzymatic reaction. However, in the ternary complex the alanine side chain may be held in place so that the complication of the equilibrium **2** $\rightleftharpoons$ **7** would not interfere with the direct breakdown of the initial peroxy intermediate to formylkynurenine *via* modified transition state **22.**

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1. Witkop, B. (1944) *Ann.* **556,** 103–114.
2. Hayaishi, O. (1974) in *Molecular Mechanisms of Oxygen Activation,* ed. Hayaishi, O. (Academic Press, New York), pp. 1–28.
3. Witkop, B. & Patrick, J. B. (1951) *J. Am. Chem. Soc.* **3,** 2196–2200.
4. Saito, I., Matsuura, T., Nakagawa, M. & Hino, T. (1977) *Acc. Chem. Res.* **10,** 346–352.
5. Fontana, A. & Toniolo, C. (1976) in *Fortschritte der Chemie Organischer Naturstoffe,* eds. Herz, W., Grisebach, H. & Kirby, G. W. (Springer-Verlag, New York), Vol. 33, pp. 309–449.
6. Weil, L., Gordon, W. G. & Buchert, A. R. (1951) *Arch. Biochem. Biophys.* **33,** 90–109.
7. Yoshida, Z. & Kato, J. (1954) *J. Am. Chem. Soc.* **76,** 311–312.
8. Obata, Y. & Sakamura, S. (1954) *Nippon Nogei-Kagaku Kaishi* **28,** 768–771.
9. Hara, H. (1960) *Nippon Nogei-Kagaku Kaishi* **34,** 493.
10. Sluyterman, L. A. (1962) *Biochim. Biophys. Acta* **60,** 557–661.
11. Gurnani, S., Arifuddin, M. & Augusti, K. T. (1966) *Photochem. Photobiol.* **5,** 495–505.
12. Savige, W. E. (1971) *Aust. J. Chem.* **24,** 1285–1293.
13. Benassi, C. A., Scoffone, E., Galiazzo, G. & Iori, G. (1967) *Photochem. Photobiol.* **6,** 857–866.
14. Spikes, J. D. & MacKnight, M. L. (1970) *Ann. N.Y. Acad. Sci.* **171,** 149–162.
15. Nakagawa, M. & Hino, T. (1977) *Yuki Gosei Kagaku Kyokaishi* **35,** 42–56.
16. Witkop, B. (1956) *J. Am. Chem. Soc.* **78,** 2873–2882.
17. Kissman, A., Ek, H., Patrick, J. B. & Witkop, B. (1952) *Experientia* **8,** 36–40.
18. Hamilton, G. A., Feigelson, P. & Brady, F. O. (1974) in *Molecular Mechanisms of Oxygen Activation,* ed. Hayaishi, O. (Academic Press, New York), pp. 405–451.
19. Sundberg, R. J. (1970) in *Chemistry of Indoles* (Academic Press, New York), pp. 289–296.
20. Hamilton, G. A. (1969) *Adv. Enzymol.* **32,** 55–96.
21. Nakagawa, M., Kaneko, T., Yoshikawa, K. & Hino, T. (1974) *J. Am. Chem. Soc.* **96,** 624–625.
22. Nakagawa, M., Yoshikawa, K. & Hino, T. (1975) *J. Am. Chem. Soc.* **97,** 6496–6501.
23. Nakagawa, M., Okajima, H. & Hino, T. (1976) *J. Am. Chem. Soc.* **98,** 635–637.
24. Nakagawa, M., Okajima, H. & Hino, T. (1977) *J. Am. Chem. Soc.* **99,** 4424–4429.
25. Savige, W. E. (1975) *Aust. J. Chem.* **28,** 2275–2287.
26. Karle, J. & Karle, I. L. (1966) *Acta Crystallogr.* **21,** 849–859.
27. Denney, D. B. & Denney, D. G. (1957) *J. Am. Chem. Soc.* **79,** 4806–4808.
28. Hedaya, E. & Winstein, S. (1967) *J. Am. Chem. Soc.* **89,** 1661–1672.
29. van der Plas, H. C. (1973) *Ring Transformations of Heterocycles* (Academic Press, New York), Vol. 1, pp. 245–246.
30. Cohen, L. A. & Witkop, B. (1955) *J. Am. Chem. Soc.* **77,** 6595–6600.
31. Dewar, M. J. S. & Thiel, W. (1974) *J. Am. Chem. Soc.* **97,** 3978–3986.
32. Savige, W. E. & Fontana, A. (1976) *J.C.S. Chem. Commun.,* 600–601.
33. Nakagawa, M. & Hino, T. (1970) *Tetrahedron* **26,** 4491–4503.
34. Witkop, B., Patrick, J. B. & Kissman, H. (1952) *Ber. Deutsche Chem. Ges.* **85,** 949–977.