Tetrahedron Vol. 46, No. 2, pp. 661-670, 1990
Printed in Great Britain

0040-4020/90 $3.00+.00

MODEL STUDIES FOR INSECT PROTEIN SCLEROTIZATION: OXIDATIVE LOSS OF THE SIDE CHAIN FROM 4-SUBSTITUTED CATECHOLS

Stephen G. Pyne*, Roger J.W. Truscott*,
Karin Maxwell, M. Carmen Morales, Bernadette C. Walsh and Barbra L. Wynn,
Department of Chemistry, University of Wollongong,
P.O. Box 1144, Wollongong, N.S.W. 2500, Australia.

(*Received in UK* 12 *October* 1989)

Abstract: The oxidation of several 4-substituted catechols in aqueous solution, pH7 in the presence of aniline results in the formation of 4,5-dianilino-1,2-benzoquinone 2 and the anil of 2, (3). The C-4 substituent is efficiently cleaved and mechanisms are proposed to account for this phenomenon based on HPLC and GC studies and the formation of N-methylaniline when these reactions were performed in the presence of sodium cyanoborohydride. The evidence suggests that the initial step involves the addition of aniline to the catechol C-4 side chain.

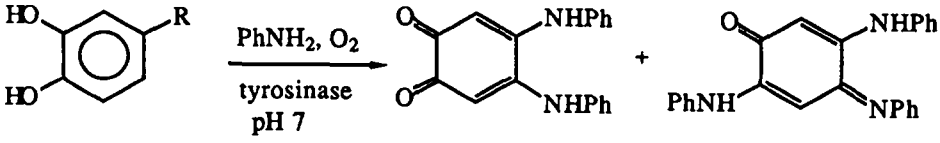
Numerous 4-substituted 1,2-dihydroxybenzenes have been implicated in the crosslinking of protein during insect sclerotization,[1] a process in which the new insect cuticle is hardened and darkened. These catechol compounds are normally excreted from insect glands during ecdysis along with oxidase enzyme(s). Mechanisms that have been suggested for insect protein tanning include: (i) quinone tanning,[2] where it has been reasoned that catechols are first oxidized to o-quinones which may then cross-link protein possibly through lysine and histidine residues; (ii) β-sclerotization,[3] a process assumed to involve crosslinking of proteins through the α and β carbons of the side chain of N-acetyldopamine; (iii) quinone methide tanning,[4] resulting from initial protein modification via p-quinone methides that arise from the tautomeric rearrangement of o-quinones. The modification of biopolymers by catechols has recently been reviewed by Peter.[5]

As part of a program to investigate the molecular mechanism of insect protein tanning we have performed model reactions with various catecholic tanning agents. The catechols 1b-f were individually oxidized using the enzyme tyrosinase at pH 7 in the presence of aniline as a model for the nucleophilic groups of lysine and histidine residues of proteins. These residues have been recently implicated in insect protein tanning from solid state NMR studies.[6]

Results and Discussion

The tyrosinase catalysed oxidation of 1b-f at pH 7 in the presence of aniline (5 molar equivalents) gave a mixture of 4,5-dianilino-1,2-benzoquinone 2 and the anil 3 (Table 1). These two compounds have been isolated and characterized previously by us[7] and others[8] from the oxidation of catechol 1a (R=H) in the presence of aniline. The C-4 substituent of 1b-f is thus efficiently cleaved under the oxidative reaction conditions.

Table I Oxidation of catechols 1b-f in the presence of aniline



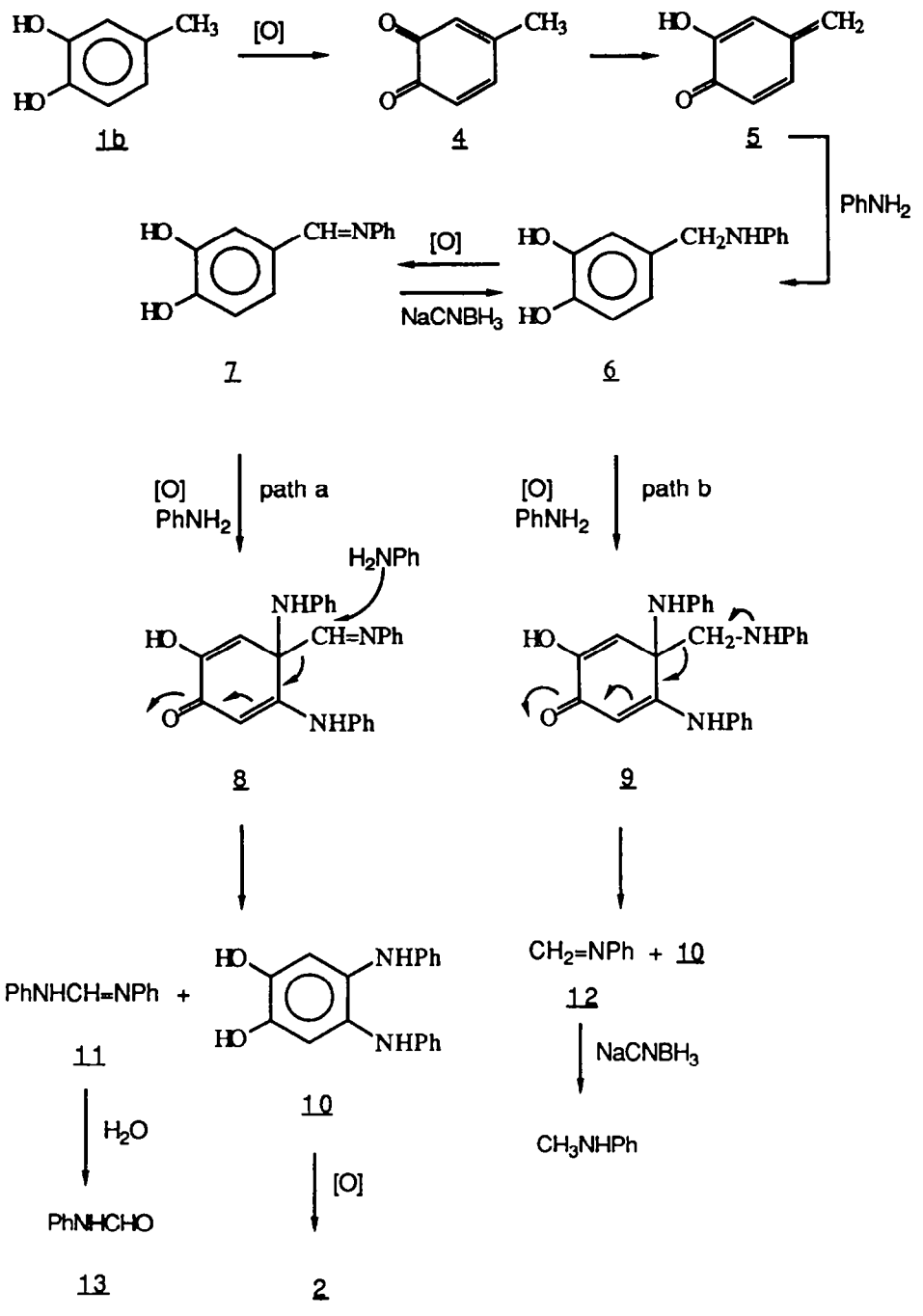
1 2 3

Catechol	2 (%)	3 (%)
1b R = CH_3	20	62
1c R = CH_2COOH	9	66
1d R = CH_2OH	13	78
1e R = COOH	49	32
1f R = CHO	24	34

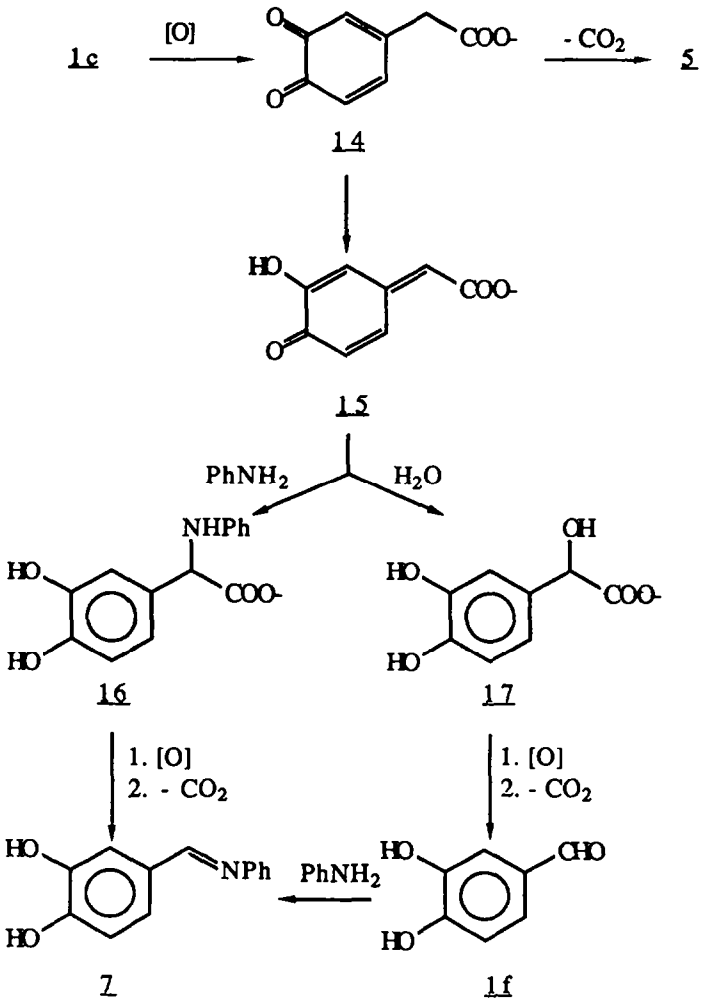
A similar phenomenon has been observed in the reaction of 4-alkyl-o-quinones with amines in non-aqueous media.[8] For example, the reaction of 4-methyl-o-quinone 4 with aniline gave 2 and 3.[8] A mechanism was proposed which involved initial rearrangement of the o-quinone 4 to the 2-hydroxy p-quinone methide 5 which underwent conjugate addition of aniline (5 → 6) and subsequent loss of the C-4 side chain as formaldehyde anil 12 (Scheme I), and formation of 10. Oxidation of 10 then yields 2 and finally 3 by further reaction with aniline.

To study the mechanism of side chain loss we have performed enzymic oxidations of 1b and 1c in the presence of sodium cyanoborohydride ($NaCNBH_3$). It was anticipated that the reactive anil 12 could be trapped *in situ* by reduction to N-methylaniline. Oxidation of 1b or 1c in the presence of aniline and $NaCNBH_3$ (2 molar equivalents) was found to give a mixture of 2 and 3 and N-methylaniline, the latter compound being quantified by GC using N-ethylaniline as an internal standard (Table II).

Scheme I


HO
CH3
[O]
O
CH2
1b
4
5
PhNH2
CH=NPh
CH2NHPh
NaCNBH3
7
6
path a
path b
H2NPh
NHPh
8
9
CH2=NPh + 10
12
NaCNBH3
CH3NHPh
PhNHCH=NPh +
11
H2O
PhNHCHO
13
10
2

Scheme II



Scheme III

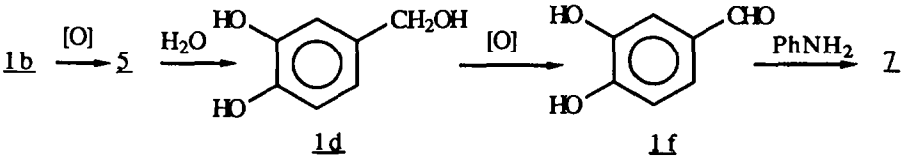


Table II Oxidation of 1b and 1c in the presence of aniline and $NaCNBH_3$

Substrate	2 + 3	N-methylaniline
1b	38%	10%
1c	67%	27%

A significant amount of N-methylaniline was formed under the reaction conditions, however the correlation between the amount of 2 and 3 and that of N-methylaniline was only modest. These results suggest that, under the above reaction conditions, the reaction pathway proposed by Tedder et al[8] for the loss of the side chain from quinones is plausible. (See Scheme I) Competing reaction pathways may operate and furthermore it is not clear whether the presence of $NaCNBH_3$ affects the course of these reaction pathway(s). We have shown that $NaCNBH_3$ is capable of completely inhibiting the catalytic activity of tyrosinase but that under these conditions the products 2 and 3 arise from autoxidation of 1.

To further elucidate the reaction mechanism(s) we have monitored the oxidation of 1b and 1c in the absence of $NaCNBH_3$ and in the presence and absence of aniline by HPLC. Using a similar analytical technique, other workers[9] have detected the formation of 1d and 1f and 3,4-dihydroxy-mandelic acid 17 from the enzymatic oxidation of 1b and 1c in water (pH 7) respectively. We observed similar results, (Figure 1 and Figure 2) however the initial oxidation product of 4-methyl catechol, 1d, could not be detected. When the same experiments were performed in the presence of aniline the imine 7 was detected from both catechols (Figures 3 and 4). The HPLC solvent program was optimized to ensure resolution of 7 from other possible intermediates, for example, 6, 1d and 1f. An authentic sample of 7 was prepared from 1f and aniline, while reduction of 7 gave 6, a proposed intermediate in Scheme I.

The major peak (retention time ca 6 min, Figure 4) after 1 min of oxidation, was collected from the HPLC and was shown to be identical to an authentic sample of the imine 7 by MS, UV and HPLC retention time. In Scheme I and Scheme II mechanisms are proposed to account for the formation of 7 from both 4-methylcatechol and dihydroxyphenyl acetic acid. The mechanistic pathway shown in Scheme I ultimately results in the loss of the C-4 side chain as N,N'-diphenylformamidine 11 which would suffer rapid hydrolysis under the reaction conditions to give formanilide 13. We have detected formanilide by GC, albeit in low yield (2-5%), from the oxidation of 1b and 1c in the presence of aniline. Formanilide was found to be unstable under the reaction conditions. In a control experiment a standard solution of formanilide in pH 7 buffer was stirred in the presence of oxygen and tyrosinase over the

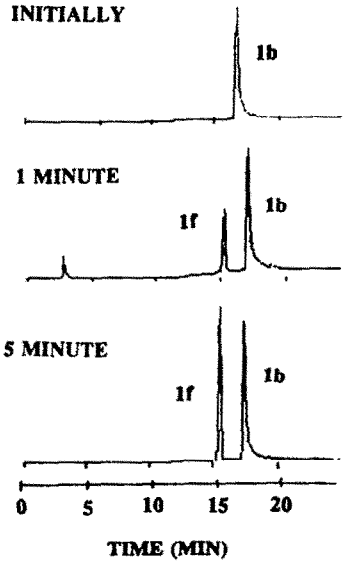


FIGURE ONE:
Time Course of the oxidation of 1b by tyrosinase using Solvent Program 1.

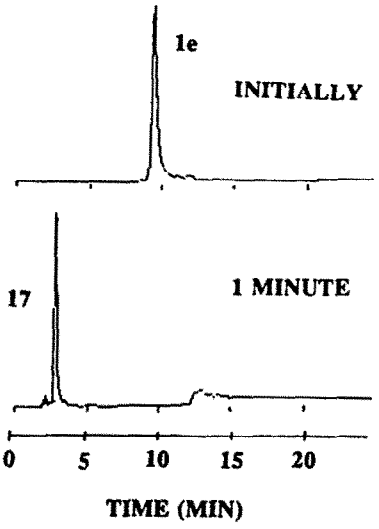


FIGURE TWO:
Time Course of the oxidation of 1e by tyrosinase using Solvent Program 1.

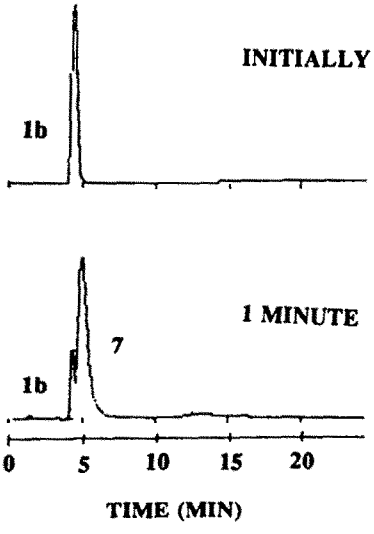


FIGURE THREE:
Time Course of the oxidation of 1b by tyrosinase in the presence of aniline using Solvent Program 2.

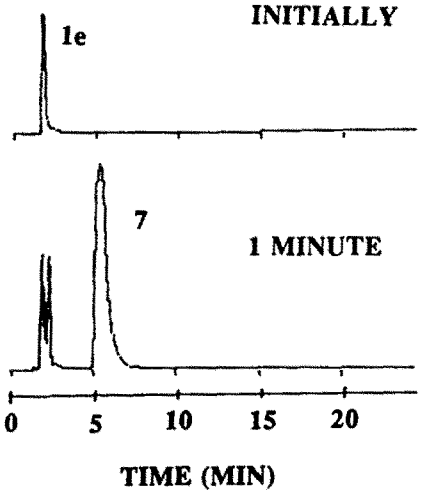


FIGURE FOUR:
Time Course of the oxidation of 1e by tyrosinase in the presence of aniline using Solvent Program 2.

reaction time period (66 hr). The decomposition of formanilide was monitored by quantitative GC and after 66 hr, 70% decomposition of formanilide was observed.

Conclusion

In conclusion a variety of sclerotizing catechols have been shown to undergo loss of the C-4 side chain during oxidation in the presence of aniline to give 2 and 3. In the case of 4-methylcatechol 1b, two competing mechanistic pathways may be plausible. The oxidation of 1b in the presence of $NaCNBH_3$ gives N-methylaniline supporting a mechanistic scheme similar to that proposed by Tedder[8] (path b, Scheme I). When this reaction, in the absence of $NaCNBH_3$, was monitored by HPLC the imine 7 could be detected as an intermediate suggesting an alternative or competing reaction pathway (Scheme I). The $NaCNBH_3$ may cause reduction of 7 to 6 and 8 to 9, forcing the reaction to proceed via path b. In the case of 1b, 1c and 1d, the aldehyde 1f may be a common intermediate if water can compete effectively with aniline in these reaction pathways (Scheme II and III). It is not possible at this stage to assess the relative importance of these suggested mechanistic pathways. Curent work is aimed at the synthesis of compound 16 and an examination of its mechanism of oxidation (Scheme II).

Although different species of insects have their own unique catechols for sclerotization these results suggest that a common structural crosslink may be present in insect cuticular proteins.

Experimental

General Procedures: Spectroscopic data was determined as previously described.[7] A Varian model 3700 gas chromatograph equipped with a flame ionization detector and BP5 (25 μm) column (SGE, Australia) was used for gas chromatography. High Performance Liquid Chromatography was carried out on an ICI HPLC SYSTEM, using a RESOLVE C18 Reverse Phase Radial-PAK Cartridge (8mm x 10cm x 5mm, WATERS). The mobile phase consisted of eluent A, 40 mM sodium acetate buffer, adjusted to pH 5.0 with glacial acetic acid and eluent B, acetonitrile. The eluents were degassed by vacuum filtration prior to use. The column was eluted isocratically for 5 minutes.

Synthesis of Catechols

Catechols 1b, 1c, 1e and 1f were purchased from Sigma Chemical Company. 3,4-dihydroxybenzyl alcohol 1d was prepared from reduction of 1f as previously described,[10] mp 135°C, lit.[10] 137°C. The imine 7 (mp 170°; ^1H NMR (d^6 - DMSO), 8.36 (s, 1H), 7.38-7.13 (m, 7H), 6.83 (d, J = 8.2 Hz, 1H); ^{13}C NMR (d^6 - DMSO), 114.2, 115.4, 120.6, 122.3,

125.0, 127.9, 128.9, 145.5, 149.1, 151.8, 156.9, 159.9; CIMS 214 ($M+H^+$, 100%)) was prepared from grinding an intimate mixture of 1f (10.7 mmol), aniline (10.7 mmol) and chloroform (10 ml) in a mortar and pestle. The crude product was recrystallized from aqueous ethanol (1.0g, 44% yield). Reduction of 7 (0.5g) in ethanol (20 ml) with sodium borohydride (0.2g) at 25° for 2 hr gave 1e (.15g after recrystallization from aqueous ethanol (1:1)), mp 216°C, ^1H NMR (d^6 - DMSO), 4.06 (s, 2H), 6.63 (m, 8H); ^{13}C NMR (d^6 - DMSO) 46.6, 112.5, 114.7, 115.2, 115.9, 118.0, 128.5, 143.8, 144.9, 148.0).

General Catechol Oxidation Method

To a stirred solution of the catechol (0.5g) and aniline (2.20g) in 50 ml of phosphate buffer (pH 7) was added 1 mg of tyrosinase. The solution was oxygenated (1 ml/sec^{-1}) for a period of sixty six hours, during which time additional tyrosinase (1 mg every twelve hours) was added to the reaction mixture. The reaction was carried out in a concial flask at room temperature for 66 hrs. The reaction mixture was filtered and the precipitate collected. The precipitate was then evacuated under pressure to remove the last traces of aniline followed by chromatographic separation on a bed (8cm x 4cm) of silica gel 0.063-0.20 mm (Merck). Elution with chloroform and then evaporation of solvent gave the major products 2 and 3. Table I lists the yields of 2 and 3 with various substituted catechols.

General Catechol Oxidation Method in the Presence of $NaCNBH_3$

A solution containing the catechol 1c (0.5g), 6 molar equivalents of aniline (1.66g), 2 molar equivalents of $NaCNBH_3$ (0.37g) and 1 mg of tyrosinase in 0.2M sodium phosphate buffer (50 ml, pH 7.0) was stirred at room temperature for 66 hours. During this time, molecular oxygen was bubbled through the solution and 1 mg of tyrosinase was added every 12 hours. After 66 hours incubation, the dark brown-coloured precipitate was collected by filtration and dried *in vacuo*. The crude product was then dissolved in chloroform and purified on a silica gel column.

N-Methylaniline Determination

Following filtration of the above precipitate, 100 mg of N-ethylaniline was added to the filtrate as an internal standard. The pH was adjusted to 11, with 50% NaOH and the solution extracted with $CHCl_3$ (3 x 50 ml). The combined organic phase was then extracted with 10% HCl (3 x 50 ml). The acidic aqueous layer was adjusted to pH 11 at 0° and re-extracted with ethyl acetate (3 x 75 ml). The organic extract was dried ($MgSO_4$), filtered and evaporated. This solution was then made up to 25 ml in a volumetric flask and then analysed by GC. The amount of N-methylaniline was determined from a standard curve based on the ratio of peak

areas of N-methylaniline to N-ethylanaline (internal standard).

GC program: 50°C to 280°C at 8°C/min, 1 μl of sample injected, Retention times:

Amine	T_R
Aniline	6.3 min
N-Methylaniline	8.0 min
N-Ethylaniline	9.3 min

High Performance Liquid Chromatography Analysis

(i) General Catechol Oxidation

To a solution of catechol (5 mg) in 200 μl of phosphate buffer was added 1 mg of tyrosinase. The solution was mixed and after varying time intervals (0, 1, 2 or 5 min), it was filtered (0.45 μm membrane) and 10 ul injected on the HPLC system outlined in the general procedures.

(ii) General Catechol Oxidation in the Presence of Aniline

To a solution of catechol (5 mg) and aniline (1 mg) in 200 μl of phosphate buffer was added 1 mg of tyrosinase. The solution was mixed and after varying time intervals (0, 1, 2 or 5 min), it was filtered (0.45 μm membrane) and 10 μl injected on the HPLC system. Gradient elution consisted of isocratic elution with 25% B for 15 minutes followed by a linear gradient of 25-80% B for 15 minutes.

Acknowledgement

We thank the University of Wollongong Research Grants Committee for partial financial support.

References

1. (a) Brunet, P.C.J. Insect Biochemistry (1980), 10, 467; (b) Hopkins, T.L., Morgan, T.D., Kramer, K.J. Insect Biochemistry (1984), 14, 533.

2. (a) Pryor, M.G.M. Proc. Roy. Soc. (1940), B128, 378; (b) Pryor, M.G.M. Proc. Roy. Soc. (1940), B128, 393; (c) Pryor, M.G.M., In 'Comparative Biochemistry' (Florkin, M. Mason, H.S., Ed.), Vol. 4, pp. 371-396. Academic Press, New York.

3. Anderson, S.O., In 'Comprensive Insect Physiology Biochemistry and Pharmacology' (Kokot, G.A. and Gilbert, L.I., Ed.), (1985), Vol. 3, pp. 59-64.

4. Lipke, H., Sugumaran, M., and Henzel, W., Advances in Insect Physiology (1983), 17, 1.

5. Peter, M.G. Angew Chemie, Int. Ed. Engl. (1989), 28, 555.

6. Schaefer, J., Kramer, K.J., Garbow, J.R., Jacob, G.S., Stejskal, E.O., Hopkins, T.L., Speirs, R.D. Science, (1987), 235, 1200.

7. Manthey, M.K., Pyne, S.G., Truscott, R.J.W. Aust. J. Chem., (1989), 42, 365.

8. Horspool, W.M., Smith, P.I., Tedder, J.M. J. Chem. Soc., Perkin I (1972), 1025.

9. (a) Sugumaran, M. Lipke, H. FEBS Letters (1983), 155, 65; (b) Sugumaran, M., Lipke, H., Federation Proc. P. (1983), 42, 1828; (c) Sugumaran, M. Bioorganic Chem. (1987), 15, 194.

10. Pan, R.N. and Acheson, R.M., Biochim. Biophys. Acta. (1968), 158, 206.