

Detection of Intercatechol Cross-Links in Insect Cuticle by Solid-State Carbon-13 and Nitrogen-15 NMR

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Abstract: Solid-state NMR has been used to detect an intercatechol covalent bond in sclerotized cuticle of the tobacco hornworm (*Manduca sexta*). Rotational-echo, double-resonance (REDOR) ¹³C and ¹⁵N NMR, in combination with ¹H–¹³C dipolar modulation and ¹⁵N–¹⁵N dipolar restoration at the magic angle, were used to examine hornworm pupal exuviae labeled with β-[¹⁵N]alanine and either [β-¹³C]dopamine or [α-¹³C,¹⁵N]dopamine. The REDOR spectra showed the incorporation of *N*-β-alanyldopamine into insect cuticle by the formation of a variety of covalent bonds. One of these bonds links the terminal nitrogen of one *N*-β-alanyldopamine molecule to the β carbon of another to form an intercatechol covalent bond. This result is interpreted in terms of a novel structure for stabilized cuticle that postulates protein–catechol–catechol–protein cross-links. REDOR spectra also showed oxygen substitution at both α and β carbons of *N*-β-alanyldopamine, consistent with the possibility of intercatechol oxygen bridges in dimeric or oligomeric forms of catecholamines in the cuticle.

Introduction

β-Alanine is conjugated with dopamine to form *N*-β-alanyldopamine, the major catecholamine found in the pupal cuticle^{1–4} of the tobacco hornworm, *Manduca sexta* (Figure 1). Even though the concentration of *N*-β-alanyldopamine in pupal cuticle increases with the degree of sclerotization, less than 10% of the diphenols detected by solid-state ¹³C NMR is extractable with dilute cold acid.⁵ The majority remains a permanent part of the cuticular residue. Eighty percent of the radioactivity from [8-¹⁴C]-*N*-β-alanyldopamine remains insoluble even after heating at 115 °C in 6 M HCl for 24 h.⁶ Prolonged heating of sclerotized cuticle in concentrated base generates chitin, but with diphenols and histidyl nitrogens still attached.⁷ These results suggest that diphenols such as *N*-β-alanyldopamine form covalent bonds with protein or chitin (or both) in the insect exoskeleton.

The results of solid-state NMR experiments performed on *M. sexta* pupal cuticle labeled by ¹³C and ¹⁵N emphasize the central role of *N*-β-alanyldopamine in cuticle biochemistry. Two kinds of nitrogen–carbon covalent bonds linking an imidazole nitrogen of histidyl residues to *N*-β-alanyldopamine already have been established by NMR: one to dopamine ring carbons⁷ (by double-cross-polarization) and the other to dopamine β carbons⁸

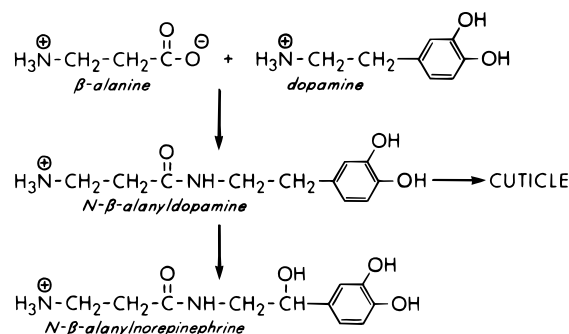


Figure 1. Pathway for the synthesis in insects of *N*-β-alanyldopamine from β-alanine and dopamine. *N*-β-Alanylnorepinephrine is an additional minor reaction product.

(by rotational-echo double resonance, REDOR). Oxygen-substituted β carbons of *N*-β-alanyldopamine are also evident in the ¹³C NMR spectra. A fourth modification of *N*-β-alanyldopamine involves substitution reactions at the primary amino group derived from β-alanine to form secondary amines.⁸ This result suggests that cross-linking occurs at the amino terminus of *N*-β-alanyldopamine, but the identification of the carbon or carbons involved had not been made previously. In this paper, we introduce two new highly selective combination REDOR NMR experiments to detect the incorporation into *M. sexta* pupal cuticle of β-[¹⁵N]alanine and either [β-¹³C]dopamine or [α-¹³C,¹⁵N]dopamine. The results of these experiments establish unambiguously the formation of intercatechol covalent bonds and lead to the proposal of a novel type of cross-link for cuticle stabilization.

Experimental Section

Insect Rearing and Cuticle Isotopic Labeling. *M. sexta* larvae were reared at 27 °C as described by Bell and Joachim.⁹ The photoperiod was 16 h of light and 8 h of dark. Larvae were selected

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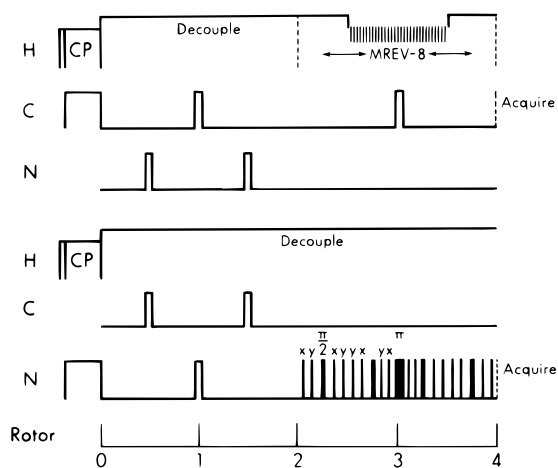


Figure 2. Pulse sequences for rotational-echo double resonance (REDOR) combined with dipolar rotational spin-echo NMR (DRSE, second half of top sequence) and dipolar restoration at the magic angle (DRAMA, second half of bottom sequence). REDOR-DRSE is used to count the number of protons directly bonded to a ^{13}C that also is directly bonded to ^{15}N . REDOR-DRAMA is used to measure ^{15}N dipolar coupling to an ^{15}N that is directly bonded to ^{13}C . Both illustrations are for four rotor cycles.

for isotopic labeling on day 4 of the fifth larval stadium during the wandering phase of development. Isotopically labeled compounds (99 atom % in ^{13}C and ^{15}N) were obtained from MSD Isotopes (Montreal). β - ^{15}N Alanine (10 mg) and either $[\beta$ - $^{13}\text{C}]$ dopamine or $[\alpha$ - $^{13}\text{C},^{15}\text{N}]$ -dopamine (5 mg) were dissolved in 0.05–0.2 mL of water, and the pH was adjusted to 6.8. After being filtered through a 0.5- μm Millex-PF filter (Millipore, Bedford, MA), the solutions were injected into ice-chilled larvae through an abdominal proleg. Pupal exuviae were collected after adult ecdysis, rinsed in distilled water, air-dried, mixed with dry ice, and ground into a powder at -70°C with a mortar and pestle. Similar procedures were followed for three other double labelings using (i) $[\alpha$ - $^{13}\text{C}]$ dopamine and β - ^{15}N alanine, (ii) $[\alpha$ - $^{13}\text{C}]$ -dopamine and $[\text{ring-}^{15}\text{N}_2]$ histidine, and (iii) $[\beta$ - $^{13}\text{C}]$ dopamine and $[\epsilon$ - $^{15}\text{N}]$ -lysine.

NMR Spectrometer. Cross-polarization, magic-angle spinning (CPMAS) Hahn-echo ^{13}C NMR spectra were obtained at room temperature at 50.3 MHz, and the corresponding ^{15}N NMR spectra at 20.3 MHz. The single, 13-mm diameter, radio-frequency coil was connected by a low-loss transmission line to a triple-resonance tuning circuit. One-kilowatt ^1H -, ^{13}C -, and ^{15}N -tuned transmitters produced maximum radio-frequency-field amplitudes of 100, 50, and 40 kHz, respectively. Cross-polarization transfers were performed at 38 kHz, and proton dipolar decoupling at 100 kHz. Rotors with 1-g sample capacities were made from ceramic (zirconia) barrels fitted with plastic (Kel-F) end caps and supported at both ends by air-pumped journal bearings. In these experiments, 300-mg samples were positioned in the center of the rotor by Kel-F barrels fitted with threaded caps.

REDOR. The pulse sequence used for REDOR experiments^{10,11} is illustrated in Figure 2 (first half of each sequence). The single ^{13}C π pulse in the middle of the REDOR carbon-magnetization dephasing period (top left) refocuses all isotropic chemical shifts at the start of data acquisition. In the absence of any ^{15}N pulses, the echo that forms results in S_0 , the REDOR full-echo spectrum, which, for this sequence, is also the CPMAS Hahn-echo spectrum. Application of phase-alternated¹¹ nitrogen π pulses at every half rotor cycle causes a net dephasing¹¹ of the transverse magnetization of those carbons dipolar coupled to ^{15}N . The resulting spectrum, S , is diminished in intensity. The REDOR difference spectrum ($\Delta S = S_0 - S$) arises only from those carbons that are dipolar coupled to nitrogen.¹¹ REDOR dephasing was accumulated over four, six, or eight rotor cycles with magic-angle spinning at 3205 Hz. For this spinning speed, a four to eight rotor-cycle dephasing period is optimal for detection of directly bonded ^{13}C – ^{15}N pairs, for which the REDOR dephasing is approximately 100% of

the full-echo signal. For ^{13}C and ^{15}N separated by 4 Å, the four rotor-cycle dephasing is 0.4% and the eight rotor-cycle dephasing is 1.6% of the full-echo signal.¹¹ REDOR with ^{15}N observation and ^{13}C -dephasing π pulses was performed in a manner analogous to ^{13}C -observed REDOR. Because the rate of dephasing of sidebands differs from that of centerbands,¹¹ residual spinning side bands should not be suppressed in REDOR experiments.

DRSE. Carbon dipolar line shapes were characterized by a version of dipolar rotational spin-echo (DRSE) ^{13}C NMR extended over two rotor cycles.¹² This is a two-dimensional experiment in which, during the additional time dimension, carbon magnetization is allowed to evolve under the influence of ^1H – ^{13}C coupling, while ^1H – ^1H coupling is suppressed by homonuclear multiple-pulse semi-windowless MREV-8¹³ decoupling (Figure 2, second half of top sequence). The cycle time for the homonuclear decoupling pulse sequence was 33.6 μs , resulting in decoupling of proton–proton interactions as large as 60 kHz. Sixteen MREV-8 cycles fit exactly into two rotor periods with magic-angle spinning at 3720 Hz. An 8-point Fourier transform of the time dependence of the intensity of any peak resolved by magic-angle spinning (and selected by REDOR dephasing) yields a dipolar spectrum, scaled by the MREV-8 decoupling and broken up into sidebands by the spinning. The DRSE experiment was calibrated by matching the calculated C–H dipolar sideband spectrum (with a room-temperature MREV-8 scale factor¹⁴ of 0.39) for polycrystalline 1,4-dimethoxybenzene. REDOR-selected dipolar spectra were obtained by difference: the dipolar evolution preceded by four rotor cycles with ^{13}C π pulses and ^{15}N π pulses (Figure 2, top) was subtracted from the dipolar evolution preceded by four rotor cycles with ^{13}C π pulses but without ^{15}N π pulses.

DRAMA. Dipolar restoration at the magic angle¹⁵ (DRAMA) uses rotor-synchronized 90° pulses to dephase the magnetization of isolated homonuclear pairs of dipolar-coupled spin- $1/2$ nuclei. The full DRAMA sequence¹⁶ is shown in Figure 2 (second half of bottom sequence). The π pulses at the completion of the odd-numbered rotor cycles refocus isotropic chemical shifts at the completion of the even-numbered rotor cycles. These π pulses are phase alternated following the XY8 scheme¹⁷ (xyxyxyxy). Therefore, a complete phase cycle requires 16 rotor cycles. The phase of the leading pulse of the XY8 sequence is determined by the ^{15}N -spin quadrature routing.

The frequency-offset dependence of DRAMA caused by chemical-shift tensors (and 1–2 kHz isotropic chemical-shift differences) is removed using eight equally spaced π pulses per rotor cycle.¹⁶ These pulses are placed at 1/16, 3/16, 5/16, ... of the rotor period (T_r). The phases of these π pulses also are alternated according to the XY8 scheme. Phase accumulations at the spinning frequency and twice the spinning frequency from chemical-shift interactions cancel for an isotropic powder under the four sign reversals created in each half rotor cycle by the eight π pulses.¹⁶ Two 90° pulses per rotor period are placed at 1/4 and 3/4 T_r for maximum dephasing. These pulses all have the same phase, which is determined by the ^{15}N quadrature routing. The DRAMA-dephased echo that forms at the completion of even numbers of rotor cycles using the sequence of Figure 2 (second half of bottom sequence) is S . A rotor-synchronized Hahn echo is produced on even-numbered rotor cycles by a refocusing π pulse following the completion of odd-numbered cycles (omitting the four $\pi/2$ pulses). The DRAMA ^{15}N full echo that forms at the completion of even numbers of rotor cycles in the absence of ^{15}N $\pi/2$ pulses is S_0 . The ^{15}N DRAMA difference is $\Delta S = S_0 - S$. DRAMA was calibrated for ^{15}N – ^{15}N determinations by measurement of the known two-bond distance in $[2$ - ^{13}C , 1,3- $^{15}\text{N}_2]$ allantoin. The combination REDOR-DRAMA experiment used a double difference in which four kinds of spectra were

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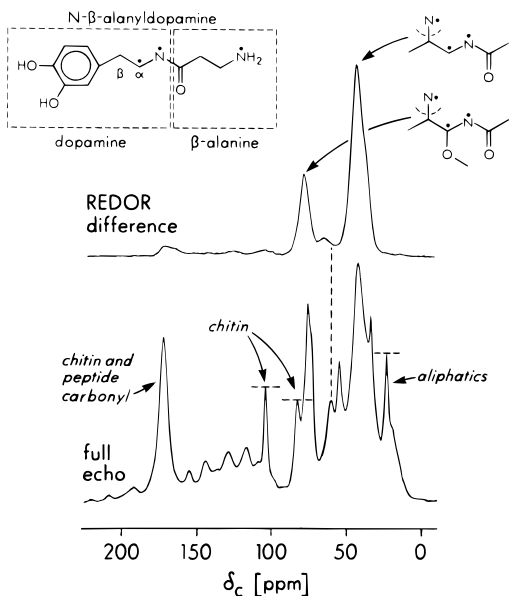


Figure 3. REDOR ^{13}C NMR spectra of *M. sexta* pupal cuticle labeled by β - ^{15}N alanine and $[\alpha$ - $^{13}\text{C},^{15}\text{N}]$ dopamine after six rotor cycles of dephasing with magic-angle spinning at 3205 Hz. The REDOR difference spectrum (top) arises exclusively from ^{13}C directly bonded to ^{15}N . The line assignments in the REDOR difference spectra are for the carbons in the structures on the right marked by the solid circles. The dotted arcs in the structures indicate that the assignment is independent of whether the attached groups are present. Line assignments for the major peaks in the full-echo ^{13}C NMR spectrum are based on chemical shifts. The peak maximum for the α carbon adjacent to an oxygen-substituted β carbon in the REDOR difference spectrum (65 ppm, top) does not coincide with the 60-ppm peak maximum in the full-echo spectrum (vertical dotted line). The heights of peaks in the full-echo spectrum (bottom) arising exclusively from natural-abundance ^{13}C are indicated by horizontal dotted lines.

obtained: with and without REDOR dephasing, and with and without DRAMA dephasing. Magic-angle spinning was at 3125 Hz.

Results

Carbon-13 REDOR. The REDOR full-echo ^{13}C NMR spectrum of the pupal cuticle labeled by β - ^{15}N alanine and $[\alpha$ - $^{13}\text{C},^{15}\text{N}]$ dopamine (Figure 3, bottom) shows natural-abundance ^{13}C peaks^{7,8} from chitin and peptide carbonyl carbons (175 ppm), diphenolics (145 ppm), aromatics (130 ppm), various oxygenated carbons of chitin (105 and 85 ppm), and protein sidechain aliphatics (20 ppm). The two major REDOR difference peaks (Figure 3, top) arise from ^{13}C labels and are assigned^{7,8} to dopamine moieties unmodified at the α -carbon position (methylene-carbon peak at 41 ppm), or oxygenated at the α -carbon position (methine-carbon peak at 77 ppm). These line assignments are indicated by structural type in the inserts to Figure 3. The 41-ppm shift matches that observed for $[\alpha$ - $^{13}\text{C},^{15}\text{N}]$ dopamine itself. Both of the REDOR difference peaks are broad, indicating that nitrogen substitution at the β -carbon position is possible at a fraction of the sites (dotted arcs in structures). The minor REDOR difference peak near 65 ppm does not coincide with a major full-echo peak. This peak and all other minor REDOR difference peaks are due to natural-abundance ^{13}C directly bonded to scrambled ^{15}N label from catabolized β -alanine. The ^{13}C and ^{15}N labels of dopamine do not scramble.⁸

The REDOR full-echo ^{13}C NMR spectrum of the pupal cuticle labeled by β - ^{15}N alanine and $[\beta$ - $^{13}\text{C}]$ dopamine (Figure 4, bottom left) has natural-abundance ^{13}C peak intensities at 175, 105, 85, and 20 ppm (horizontal dotted lines), similar to those

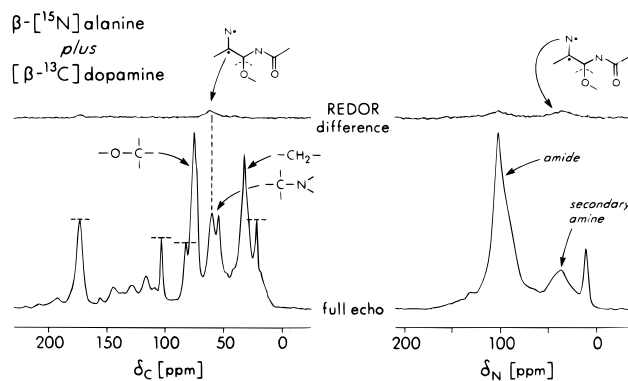


Figure 4. REDOR ^{13}C NMR spectra (left) and ^{15}N NMR spectra (right) of *M. sexta* pupal cuticle labeled by β - ^{15}N alanine and $[\beta$ - $^{13}\text{C}]$ dopamine after eight (left) or four (right) rotor cycles of dephasing with magic-angle spinning at 3205 Hz. The REDOR difference spectra arise exclusively from ^{13}C - ^{15}N directly bonded pairs. The line assignments in the REDOR difference spectra are for the carbon (top left) and nitrogen (top right) marked by the solid circles. The dotted arcs in the structures indicate that the assignment is independent of whether the attached groups are present. Line assignments for the major high-field peaks in the full-echo ^{13}C NMR spectrum are based on chemical shifts. The heights of peaks in the full-echo ^{13}C NMR spectrum (bottom, left) arising exclusively from natural-abundance ^{13}C are indicated by horizontal dashed lines.

observed in Figure 3. The ^{13}C label appears primarily in peaks near 80, 60, and 30 ppm (arrows). The 80-ppm peak corresponds to oxygen substitution at the β carbon, the 60-ppm peak to nitrogen substitution at the β carbon (with or without oxygen substitution at the α carbon), and the 30 ppm peak to no substitution at either the α or β carbons of dopamine. A comparison of Figure 4 and the natural-abundance spectrum of sclerotized cuticle in ref 8 shows that the intensity of the CHN peak at 60 ppm and that of the CH_2 peak at 40 ppm are increased by about the same amount by ^{13}C labeling. Thus, half of the ^{13}C label incorporated in non-oxygenated β carbons appears in the 60-ppm nitrogen-substituted carbon peak. The 80-ppm oxygenated β -carbon peak of Figure 4 (lower left) is bigger than anticipated based on the α - ^{13}C REDOR difference peak (65 ppm, Figure 3, top) associated with oxygen substitution at the β carbon. This result indicates that oxidation at the β -carbon site may involve further metabolism and that such sites are no longer exclusively part of a dopamine moiety.

The only REDOR difference due to a ^{13}C - ^{15}N labeled pair for the pupal cuticle labeled by β - ^{15}N alanine and $[\beta$ - $^{13}\text{C}]$ -dopamine appears at 60 ppm (Figure 4, top left). The integrated $\Delta S/S_0$ for this peak is approximately 30–40%, where the determination of S_0 is based on the comparison of peak intensities at 60 ppm arising from natural-abundance ^{13}C (Figure 3) and ^{13}C label (Figure 4), calibrated by natural-abundance peak intensities free from contributions from labels in both spectra (horizontal dotted lines).

No observed REDOR difference signals could be assigned to ^{13}C - ^{15}N directly bonded pairs of labels in the pupal cuticle double labeled using (i) $[\alpha$ - $^{13}\text{C}]$ dopamine and β - ^{15}N alanine, (ii) $[\alpha$ - $^{13}\text{C}]$ dopamine and $[\text{ring-}^{15}\text{N}_2]$ histidine, and (iii) $[\beta$ - $^{13}\text{C}]$ -dopamine and $[\epsilon$ - $^{15}\text{N}]$ lysine (spectra not shown). These null results, together with the ^{13}C chemical shifts and REDOR results of Figures 3 and 4 and ref 8, indicate that oxygen substitution of the dopamine moiety of catecholamines in insect cuticle occurs at both the α and β carbons, whereas nitrogen substitution occurs only at the β carbon.

Nitrogen-15 REDOR. Most of the ^{15}N signal intensity in Figure 4 (bottom right) is due to scrambled label from catabolized β -alanine, which is incorporated in protein and chitin

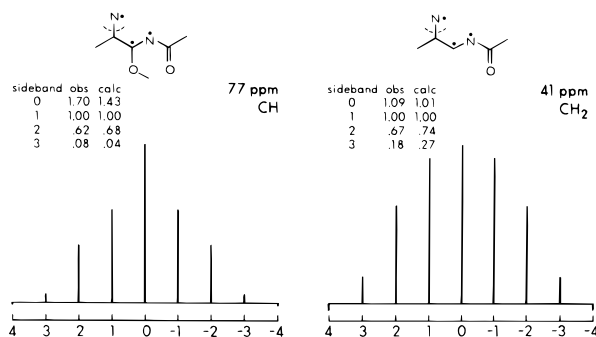


Figure 5. Dipolar sideband patterns of sp^3 ^{13}C directly bonded to ^{15}N in *M. sexta* pupal cuticle labeled by β - ^{15}N]alanine and $[\alpha$ - ^{13}C , ^{15}N]dopamine using the REDOR-DRSE pulse sequence of Figure 2 (top). The patterns have been symmetrized by zeroing of the dispersive time-domain component prior to Fourier transformation. Identification of the sp^3 carbons responsible for the REDOR difference ^{13}C NMR lines at 77 and 41 ppm is shown in Figure 3 (top, right). The REDOR selection involved four rotor cycles of dephasing, and the 1H - ^{13}C dipolar evolution two rotor cycles, with magic-angle spinning at 3720 Hz. The dotted arcs in the structures indicate that the assignment is independent of whether the attached groups are present. The dipolar sideband intensities for the 77- and 41-ppm lines are in agreement with those expected for CH and CH_2 , respectively.

as amide nitrogen. Based on signal intensity, the ^{15}N REDOR difference (with ^{13}C dephasing) for the amide-nitrogen peak is due to ^{15}N label adjacent to natural-abundance ^{13}C . Incorporation of ^{15}N label from β -alanine into histidine apparently does not occur because no 150-ppm ^{15}N REDOR difference signal is observed in Figure 4 (top right).⁸ A broad secondary-amine ^{15}N peak appears near 35 ppm, and this full-echo peak is associated with a sizable REDOR difference signal (Figure 4, top right), consistent with unscrambled nitrogen-label substitution at the ^{13}C -labeled β -carbon site of dopamine. The width of this peak indicates that oxygen substitution is possible at a fraction of the α -carbon sites (dotted arcs).

Combination REDOR Experiments. Dipolar sideband intensities from the combined REDOR-DRSE experiment on the cuticle labeled by β - ^{15}N]alanine and $[\alpha$ - ^{13}C , ^{15}N]dopamine (Figure 5) confirm the assignments of Figure 3 of the REDOR-difference peak at 77 ppm (a shift generally diagnostic of an oxygenated sp^3 carbon) to a carbon with one directly bonded hydrogen, and the peak at 41 ppm to a carbon with two directly bonded hydrogens. REDOR dephasing by ^{13}C for this same sample resulted exclusively in an amide-nitrogen signal (not shown). This is the nitrogen signal that is complimentary to the ^{13}C REDOR difference spectrum of Figure 3 (top).

Based on the comparison of calculated and observed DRAMA dephasing in the combined REDOR-DRAMA experiment (S/S_0 , Figure 6), the REDOR-selected amide ^{15}N has a dipolar coupling of 40 Hz to a second ^{15}N . A dipolar coupling of 40 Hz corresponds to a three-bond ^{15}N - ^{15}N internuclear separation. The calculated dephasing¹⁶ was done assuming that half of the REDOR-selected amide nitrogens have a single nitrogen nearest-neighbor, and that this nitrogen is 40% ^{15}N enriched. The value of 40% for the isotopic enrichment for the labeled nitrogen at the substituted β -carbon site was determined from the ^{13}C REDOR results of Figure 4 (60 ppm). The assumption that half of the REDOR-selected amide nitrogens have a nearby nitrogen neighbor is based on the estimated distribution of β - ^{13}C label between CHN and CH_2 for incorporated dopamine moieties described in the second paragraph of the ^{13}C REDOR results section. If this fraction is reduced, the calculated initial dephasing is proportionately reduced. However, the fraction of REDOR-selected amide nitrogens with a nearby nitrogen

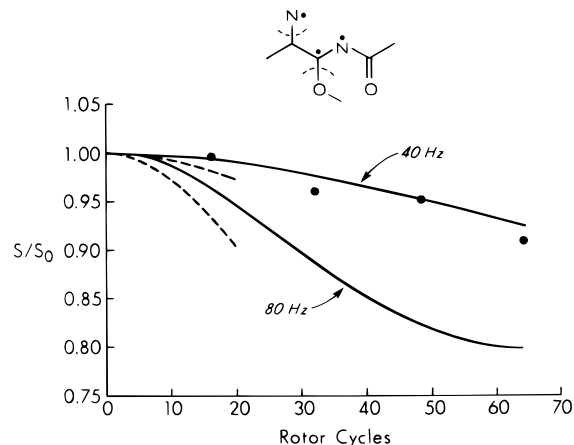


Figure 6. DRAMA ^{15}N dephasing (solid circles) for amide nitrogens directly bonded to ^{13}C in *M. sexta* pupal cuticle labeled by β - ^{15}N]alanine and $[\alpha$ - ^{13}C , ^{15}N]dopamine. The amide nitrogens were selected using the pulse sequence of Figure 2 (bottom) and four rotor cycles of ^{13}C dephasing with magic-angle spinning at 3125 Hz. The calculated dephasing (solid lines) used finite pulses and assumed that half of the REDOR-selected amide nitrogens have a single, nearest-neighbor nitrogen (40% ^{15}N enrichment) with a dipolar coupling of either 80 (two-bond separation) or 40 Hz (three-bond separation). The dotted lines show the initial DRAMA dephasing calculated assuming delta-function pulses and 80-Hz coupling (lower dotted line) or 40-Hz coupling (upper dotted line). The dotted arcs in the structure indicate that REDOR selection of the amide nitrogen is independent of substitution at the α and β carbons.

neighbor would have to be reduced by a factor of 4, to about 12%, to account for the observed 5% dephasing after 48 rotor cycles with a two-bond, 80-Hz coupling (20% dephasing expected) rather than a three-bond, 40-Hz coupling. A 12% fraction for nitrogen substitution at the β carbon is inconsistent with the observed distribution of label for dopamine moieties.

Discussion

Intercatechol Cross-Links. The secondary-amine ^{15}N REDOR difference of Figure 4 (top right) proves that nitrogen substitution of dopamine β carbons of catecholamines occurs in insect cuticle. This was, in fact, the basis for the structures drawn in the inserts to Figures 3 and 4, which show the nitrogen of the β -carbon substitution as ^{15}N labeled. We believe that this labeled nitrogen is part of an *N*- β -alanyldopamine moiety because the other ^{15}N -labeled primary amine tested did not attack the β carbon (null $[\epsilon$ - ^{15}N]lysine and $[\beta$ - ^{13}C]dopamine result), and significant routing of nitrogen label from β -alanine into histidine, which is known to attack the β -carbon site,⁸ does not occur (no signal at 150 ppm, Figure 4, right). In addition, an internal *N*- β -alanyldopamine cyclization resulting in an *intra*-catechol labeled-nitrogen substitution at the β carbon is highly unlikely because of the instability of the required seven-membered ring. A cyclization to form a plausible six-membered ring does not occur because no nitrogen substitution is observed at the α -carbon site (null β - ^{15}N]alanine and $[\alpha$ - ^{13}C]dopamine result). Therefore, we conclude that the labeled nitrogen at the β -carbon substitution site is part of an *intercatechol* covalent bond. Mobile catecholamines presumably could stabilize cuticle by cross-linking immobilized proteins to form a protein-catechol-catechol-protein structure (Figure 7). On average, only half of the β -carbon sites of a fully cross-linked cuticle would have nitrogen substitution, consistent with the structure of Figure 7, and with the number of three-bond, ^{15}N - ^{15}N couplings observed by DRAMA (Figure 6).

Catechol Dimers and Oligomers. The results of the NMR experiments of Figures 3 and 4 show that oxygen substitution

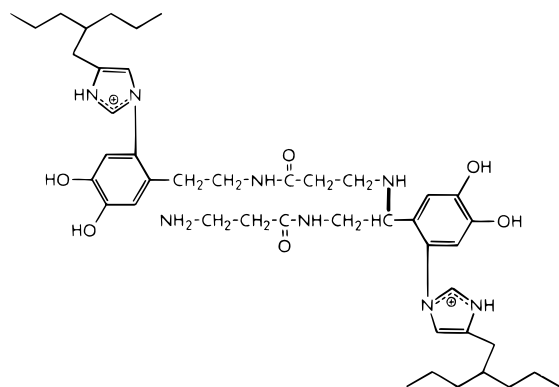


Figure 7. Proposed protein–catechol–catechol–protein cross-link (heavy line) in insect cuticle. Only two of the nitrogens of this structure are separated by three single bonds.

of the dopamine moiety of catecholamines in the sclerotized pupal cuticle of *M. sexta* occurs at both the α - and β -carbon sites. This finding supports indirect evidence^{5,18–20} for intercatechol oxygen bridges in dimers or oligomers of catechols in cuticle. We previously reported that some of the *N*- β -alanyldopamine in pupal cuticle is bound covalently by acid-labile bonds to proteins, which release *N*- β -alanyl norepinephrine (see structure at the bottom of Figure 1) upon hydrolysis.^{5,18} Therefore, ether or ester linkages between amino-acid residues in proteins, or *N*-acetylglucosamine residues in chitin, and the β carbon of *N*- β -alanyldopamine could account for oxygen substitution of this sidechain carbon. Only minor amounts of

N- β -alanyl norepinephrine occur unbound in cuticle.⁵ *N*-Acetyldopamine dimers and oligomers involving oxygen bridges between the 3,4-dihydroxyl groups of a catechol residue and the α and β carbons of another dopamine moiety have been isolated from insect cuticle or model sclerotization reaction mixtures.^{19,20} Acid hydrolysis of the dimers and oligomers yields ketocatechols, the most prominent of which is 3,4-dihydroxyphenylketoethanol. Because acid hydrolysis of either whole cuticle or purified proteins from cuticle also yields 3,4-dihydroxyphenylketoethanol,^{5,18} *N*- β -alanyldopamine dimers and oligomers probably exist in the pupal cuticle of *M. sexta*. The precursor for *N*- β -alanyldopamine dimer formation is 1,2-dehydro-*N*- β -alanyldopamine, which also has been identified as a reaction product of *N*- β -alanyldopamine and cuticle phenoxidasases and isomerases.²¹ Therefore, all of this indirect chemical evidence, as well as the direct NMR evidence described earlier, indicates that some of the oxygen substitution at α,β -carbon sites in the pupal cuticle arises from dimers or oligomers of *N*- β -alanyldopamine, which can serve as cross-links between proteins (and possibly chitin) in the matrix of the exoskeleton.

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