Detection of Cross-Links in Insect Cuticle by REDOR NMR Spectroscopy

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Abstract: Rotational-echo, double-resonance ^{13}C and ^{15}N NMR have been used to examine tobacco hornworm pupal exuviae labeled either with a combination of [β-^{13}C]dopamine and [*ring*-$^{15}N_2$]histidine or with β-[3-^{13}C,^{15}N]alanine. The spectra are consistent with the incorporation of *N*-β-alanyldopamine into insect cuticle by the formation of a variety of covalent bonds. One of these bonds links a histidyl ring nitrogen to a catechol β carbon. Other bonds involve the amino group of *N*-β-alanyldopamine. These results are interpreted in terms of a general structure for stabilized cuticle that involves cross-linking of proteins to other proteins or chitin through catecholamines.

Introduction

Rotational-echo, double-resonance (REDOR) NMR spectroscopy is a new spectroscopic technique for solids spinning at the magic angle.[1] REDOR provides a direct measure of heteronuclear dipolar coupling between isolated pairs of labeled nuclei. In a solid with a ^{13}C-^{15}N labeled pair, for example, the ^{13}C rotational echoes that form each rotor period following a ^{1}H-^{13}C cross-polarization transfer can be prevented from reaching full intensity by insertion of an ^{15}N π pulse each half-rotor period. The REDOR difference (the difference between a ^{13}C NMR spectrum obtained under these conditions and one obtained with no ^{15}N π pulses) has a strong dependence on the ^{13}C-^{15}N dipolar coupling and hence the ^{13}C-^{15}N internuclear distance.[2]

REDOR is described as double resonance even though three radio frequencies (typically ^{1}H, ^{13}C, and ^{15}N) are used because the protons are removed from the important evolution part of the experiment by resonant decoupling. The dephasing of magnetization in REDOR arises from a local dipolar ^{13}C-^{15}N field gradient and involves no polarization transfer. REDOR has no dependence on ^{13}C or ^{15}N chemical-shift tensors and does not require resolution of a ^{13}C-^{15}N coupling in the chemical-shift dimension.[2] REDOR has been used to measure a 4-Å interatomic C-N distance in a crystalline peptide[3] with an accuracy of ±0.1 Å.

REDOR is not limited to long-range distance measurements in solids. In this paper we describe the use of ^{13}C and ^{15}N REDOR NMR spectroscopy for the detection of covalent bond formation in insect cuticle labeled by a combination of L-[*ring*-$^{15}N_2$]histidine and [β-^{13}C]dopamine or by β-[3-^{13}C,^{15}N]alanine. Sclerotized insect cuticle is an insoluble, intractable, heterogeneous mix of proteins, lipids, catecholamines, polysaccharides, and water.[4,5] Sclerotization (hardening) of the cuticle has been proposed to result from catecholamines acting as dehydrating agents, protein denaturants, and precursors of quinonoid cross-linking agents for the chitin-protein matrix.[6-8] Progress has been made recently in identifying potential aromatic cross-links in intact insect cuticle by ^{1}H-^{13}C-^{15}N double-cross-polarization NMR spectroscopy.[9] The cuticle examined in the double-cross-polarization experiments was labeled with [*ring*-$^{15}N_2$]histidine and [*ring*-$^{13}C_6$]dopamine. Covalent bond formation between histidyl ring nitrogens and ring carbons of catecholamines derived from dopamine was established by observation of a C-N polarization transfer. These cross-links are consistent with an *o*-quinone sclerotization mechanism.[6-8]

Cross-links between a histidyl ring nitrogen and the β carbon of dopamine have been postulated to occur in cuticle as a result of β-sclerotization or quinone methide sclerotization.[6-8] However, double-cross-polarization NMR spectroscopy was not successful in experiments performed on insect cuticle labeled with [*ring*-$^{15}N_2$]histidine and [β-^{13}C]dopamine.[9] In those experiments, inherently slow C-N polarization transfer and fast spin-lock relaxation for the labeled dopamine carbon meant poor NMR sensitivity.[10,11] REDOR avoids both of these technical problems. The net result is an order-of-magnitude improvement in NMR sensitivity for the detection of low levels of isolated ^{13}C-^{15}N pairs. This improvement makes possible the first unambiguous identification of insect cuticle stabilization by covalent bond formation at the β carbon of dopamine.

Experimental Section

Insect Rearing and Cuticle Isotopic Labeling. The tobacco hornworm, *Manduca sexta*, was reared at 27 °C as described by Bell and Joachim.[12] The photoperiod was 16 h of light and 8 h of dark. Larvae were selected for isotopic labeling on day 4 of the fifth larval instar during the wandering phase of development. Isotopically labeled compounds (99 atom % in ^{13}C and ^{15}N) were obtained from MSD Isotopes (Montreal). [*ring*-$^{15}N_2$]Histidine (15 mg) and [β-^{13}C]dopamine (5 mg), or β-[3-^{13}C,^{15}N]alanine (10 mg), were dissolved in 0.05-0.2 mL of water and the pH adjusted to 6.8. After filtering 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, and ground into a powder (40-60 mesh) with a micromill.

NMR. Cross-polarization, magic-angle spinning ^{13}C and ^{15}N NMR spectra were obtained at room temperature at 50.3 and 20.3 MHz, re-

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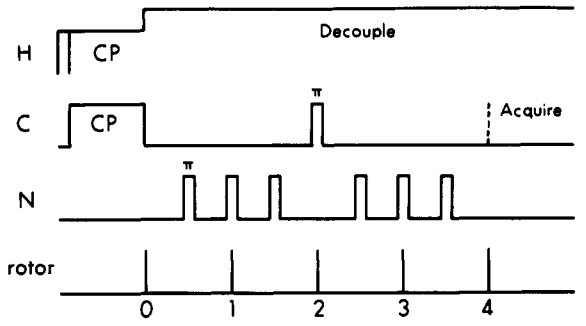

Figure 1. Rotational-echo, double-resonance (REDOR) ^{13}C NMR pulse sequence with four rotor cycles. Carbon magnetization is produced by a cross-polarization (CP) transfer from dipolar-coupled protons, which are then removed from the experiment by resonant decoupling. Application of ^{15}N π pulses every half-rotor period causes a net dephasing of transverse magnetization of carbons dipolar coupled to nitrogens. A ^{13}C π pulse replaces the ^{15}N pulse in the middle of the dephasing period and refocuses isotropic chemical shifts at the start of data acquisition. The ^{15}N pulse train is applied using *xy* phase alternation to compensate for pulse imperfections and resonance off-set effects.[14] The REDOR difference spectrum (the difference between spectra obtained with and without dephasing ^{15}N pulses) contains contributions from only those carbons that are dipolar coupled to nitrogens.

spectively. Residual spinning sidebands were suppressed by pulse techniques.[13] The single, 13-mm diameter, radio-frequency coil was connected by a low-loss transmission line to a triple-resonance tuning circuit. The 1-kW ^{1}H, ^{13}C, and ^{15}N tuned transmitters produced maximum radio-frequency-field amplitudes of 95, 80, and 40 kHz, respectively. Cross-polarization transefers were performed at 38 kHz and proton dipolar decoupling at 80 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, 150-mg samples were positioned in the center of the rotor by Kel-F barrels fitted with threaded caps.

The pulse sequence used for REDOR experiments is illustrated in Figure 1. The single ^{13}C π pulse in the middle of the REDOR carbon-magnetization dephasing period refocuses all isotropic chemical shifts at the start of data acquisition. Application of phase-alternated[14] ^{15}N π pulses every half-rotor cycle causes a net dephasing of the transverse magnetization of those carbons dipolar coupled to ^{15}N. This results in a REDOR difference spectrum (the difference between ^{13}C rotational-echo spectra with and without dephasing ^{15}N π pulses) that arises only from those carbons that are dipolar coupled to nitrogen. Weak difference spectra can be obtained reliably because the operating conditions of the observation channel do not change from scan to scan, despite, for example, an accumulation of 300 000 scans over a 4-day period. REDOR dephasing was summed over four rotor cycles with magic-angle spinning at 3.205 kHz. A four-rotor-cycle dephasing period is optimal for detection of directly bonded ^{13}C–^{15}N pairs, for which the REDOR dephasing is approximately 80% of the full-echo signal. For ^{13}C and ^{15}N separated by 4 Å, the four-rotor-cycle dephasing drops to 0.4% of the full-echo signal. REDOR with ^{15}N observation and ^{13}C dephasing π pulses was performed in a manner analogous to ^{13}C-observed REDOR. Residual spinning sidebands are not suppressed in REDOR experiments.[2]

Results

Single-Labeled Dopamine and Ring-Labeled Histidine. The natural-abundance ^{13}C NMR spectrum of pupal exuviae of tobacco hornworm shows resolved contributions from the methyl carbons of chitin (δ_C 23, Figure 2, top), the methylene carbons of lipids (δ_C 30), the oxygenated carbons of chitin (centered near δ_C 90), the diphenolic carbons of catechols (δ_C 145), and the α and carbonyl carbons of proteins (δ_C 60 and 175, respectively). The relative intensities of these peaks can be used for a semiquantitative compositional analysis of the organic content of intact tissue.[15] *M. sexta* pupal exuviae contain approximately 10% water

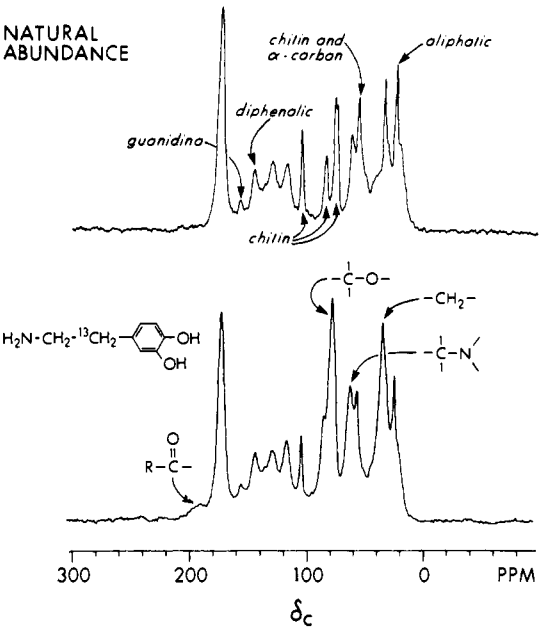

Figure 2. Cross-polarization, magic-angle spinning ^{13}C NMR spectra of tobacco hornworm pupal exuviae at natural abundance (top) and labeled by injection of [β-^{13}C]dopamine (bottom). The spectra were obtained at 50.3 MHz with total suppression of residual sidebands from the 3.205-kHz spinning. The label from dopamine appears in at least four chemical environments.

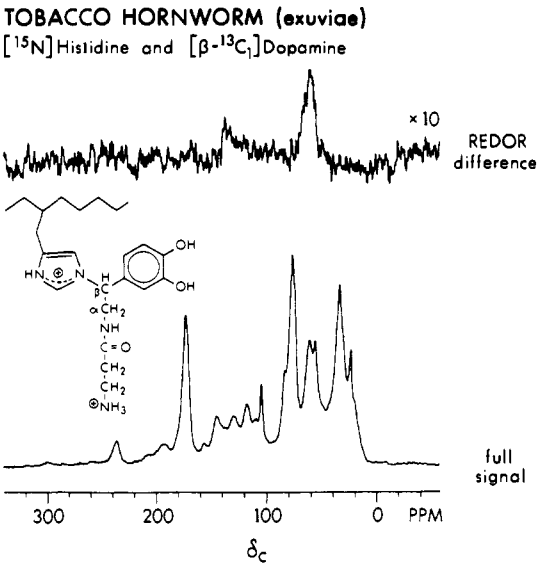

Figure 3. Rotational-echo, double-resonance ^{13}C NMR spectra of tobacco hornworm pupal exuviae labeled by injection of both [β-^{13}C]dopamine and [*ring*-$^{15}N_2$]histidine. The REDOR difference signal at 60 ppm arises from the formation of a protein–catecholamine covalent bond (insert). The weak difference signal at 120 ppm is due to natural-abundance carbons in ^{15}N-labeled histidine. The echoes were formed after four rotor cycles of evolution of transverse magnetization. Spinning sidebands have not been suppressed.

(determined by gravimetric analysis), 30% protein, 35% chitin, 20% catechols, and 5% lipid.[5]

Most of the ^{13}C label from [β-^{13}C]dopamine appears in carbons with resonances at δ_C 30, 60, and 80 which are characteristic of methylene, nitrogen-substituted, and oxygen-substituted sp^3 carbons, respectively (Figure 2, bottom). A small fraction of this label is incorporated as ketones and aldehydes (δ_C 190). In cuticle labeled by both [β-^{13}C]dopamine and [*ring*-$^{15}N_2$]histidine, only the resonance at δ_C 60 has a ^{13}C REDOR difference signal above

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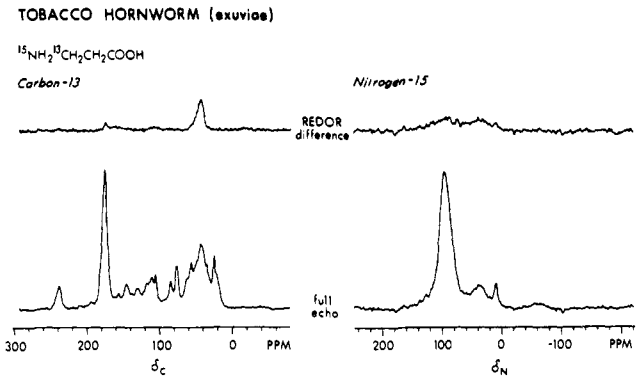

Figure 4. Rotational-echo, double-resonance ^{13}C (left) and ^{15}N (right) NMR spectra of tobacco hornworm pupal exuviae labeled by injection of β-[3-^{13}C,^{15}N]alanine. Virtually all of the ^{15}N spectrum arises from label. The echoes were formed after four rotor cycles of evolution of transverse carbon magnetization. Spinning sidebands have not been suppressed.

the ^{15}N natural-abundance level (Figure 3, top). The REDOR difference at δ_C 60 is about 10% of the full-echo signal. A difference signal of this size after only 4 rotor cycles of dephasing with spinning at 3.2 kHz is not possible for a C–N separation of more than 2.2 Å even if every ^{13}C contributing to the peak at δ_C 60 has a ^{15}N neighbor. A more plausible explanation is that 10% of the ^{13}C's are directly bonded to ^{15}N. This interpretation was confirmed by the observation that the REDOR difference decreased by a factor of only 1.9 when the number of rotor cycles with dephasing was reduced from 4 to 2, consistent with the presence of C–N dipolar coupling of the order of 1 kHz corresponding to a C–N covalent bond.[2] The REDOR difference due to weak coupling for C–N distances of 2.2 Å or more has a square-law dependence on the number of rotor cycles.[2,3] These results prove direct bonding of the β carbon of dopamine with a ring nitrogen of histidine. The REDOR difference signal intensity of Figure 3 (δ_C 60) corresponds to about 0.4 μmol of ^{13}C label. The minor difference signal at δ_C 120 arises from natural-abundance carbons in ^{15}N-labeled histidine rings.

Double-Labeled β-Alanine. Most of the ^{13}C-^{15}N label from β-alanine is scrambled. The ^{15}N enters the general nitrogen pool and is incorporated primarily as amide nitrogen in protein and chitin (δ_N 95, Figure 4, bottom right). Much of the ^{13}C label from β-alanine is not incorporated but is metabolized to CO_2 and eliminated. However, some ^{13}C label is incorporated. This is most clearly shown by a strong ^{13}C signal at 40 ppm (Figure 4, bottom left). This 40-ppm signal is observed in the spectrum of the β-alanine labeled cuticle but not in the spectrum of the natural-abundance cuticle (Figure 2, top). Moreover, the 40-ppm peak has a REDOR difference (Figure 4, top left) whose intensity is 50–75% that of the full-echo signal. A REDOR difference signal of this size demonstrates directly bonded ^{13}C–^{15}N pairs.

The integrated intensity of the ^{15}N REDOR difference signal between δ_N 0–60 of the β-alanine labeled-cuticle spectrum is about 50% of the full-echo intensity (Figure 4, right). This means that the difference signal intensity between δ_N 0–60 must arise from ^{15}N's which have been incorporated as part of unscrambled labeled pairs, and which are therefore ^{15}N's directly bonded to methylene carbons with δ_C 40. The nitrogens of these pairs with chemical shifts between 0 and 30 ppm are primary amines; those with chemical shifts between 30 and 60 ppm are secondary amines. The intensity of the ^{15}N REDOR difference signal at δ_N 95 is only 5% of the full-echo intensity, and so arises from scrambled ^{15}N.

Discussion

Chemistry at the β Carbon of Dopamine. We have used ^{13}C REDOR NMR spectroscopy to prove the formation of a ^{13}C–^{15}N covalent bond between the labeled carbon derived from [β-^{13}C]dopamine and the nonprotonated ring nitrogen of histidine (Figure 3, insert). In addition, we have measured a value of 0.1 for the associated ^{15}N $\Delta S/S$ (the ratio of the ^{15}N REDOR difference signal intensity to that of the full echo) in a 4-cycle REDOR experiment performed on the same sample with 3.2-kHz magic-angle spinning (data not shown). The ^{15}N isotopic specific activity is known to be 30% for histidyl residues in this tissue,[9] so that the expected ^{15}N $\Delta S/S$ for a directly-bonded ^{13}C–^{15}N pair is 0.3[2]. Because the observed ^{15}N $\Delta S/S$ is only a third of the expected value, only about a third of the histidyl-residue covalent bonds form through the β carbon. This conclusion is consistent with the observation reported earlier of a strong double-cross-polarization ^{13}C NMR spectrum for cuticle labeled with [*ring*-$^{15}N_2$]histidine and [*ring*-$^{13}C_6$]dopamine.[9] Approximately two-thirds of the bonds are made through the ring carbons of dopamine, and one-third through the β carbon.

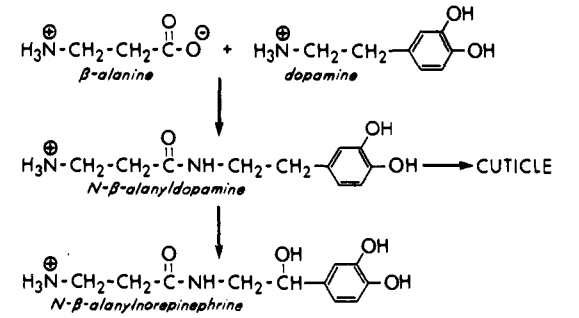

Figure 5. Pathway for synthesis of *N*-β-alanyldopamine and *N*-β-alanylnorepinephrine in insects. Double labels from β-[3-^{13}C,^{15}N]alanine are incorporated directly into cuticle via *N*-β-alanyldopamine. Nitrogen-15 from β-alanine can also be incorporated as a single label. The ^{15}N enters the general nitrogen pool and primarily appears in protein and chitin as amide nitrogen.

Conclusions about chemistry at the β carbon of dopamine based solely on NMR chemical shifts in spectra of labeled cuticle are less definitive than those based on REDOR results. Some of the signal corresponding to oxygenated carbons (δ_C 80, Figure 2, bottom) may be due to the presence of alcohols such as *N*-β-alanylnorepinephrine and *N*-acetylnorepinephrine, metabolites of *N*-β-alanyldopamine and *N*-acetyldopamine, respectively. Relatively large amounts of these alcohols, compared to the total recoverable catechols, can be extracted from *M. sexta* pupal cuticle with cold acid. This extract possibly represents hydrolytic products of weak ether or ester bonds.[16] The oxygenated-carbon signal may also contain contributions from cross-links between diphenols and chitin, as suggested by the results of extraction of cuticle with concentrated base.[9] Some of the β-carbon label of dopamine that is incorporated as ketones or aldehydes (δ_C 190, Figure 2, bottom) may correspond to ketocatechols such as 3,4-dihydroxyphenylketoethanol, which can be extracted from *M. sexta* and *Locusta migratoria* cuticle with hot dilute acid.[16,17]

Chemistry at the Terminal Nitrogen of *N*-β-Alanyldopamine. β-Alanine is conjugated with dopamine to form *N*-β-alanyldopamine, the major catecholamine[18–20] found in *M. sexta* pupal cuticle (Figure 5). 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 ^{13}C NMR spectroscopy is extractable with dilute cold acid.[16] The majority remains a permanent part of the cuticular residue. Eighty percent of the radioactivity from [8-^{14}C]-*N*-β-alanyldopamine remains insoluble even after heating at 115 °C in 6 M HCl for 24 h.[21] Prolonged heating of sclerotized cuticle in concentrated base generates chitin, but with diphenols and histidyl nitrogens still attached.[9] These results suggest that diphenols such as

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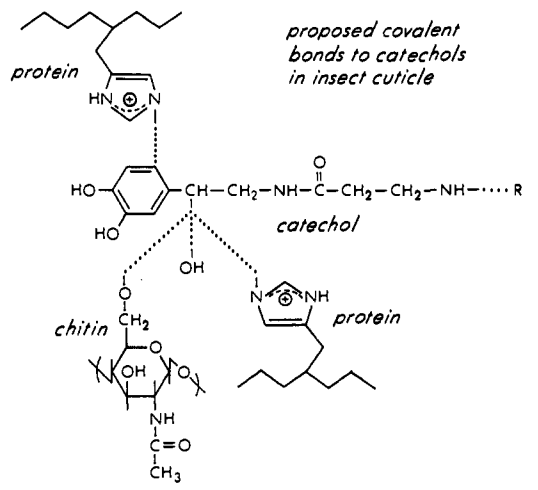

Figure 6. Proposed cross-link structure for sclerotized insect cuticle involving *N*-β-alanyldopamine. Possible cross-links are indicated by dotted lines. The covalent bonds between histidine ring nitrogens and catechol ring and β carbons have been established by NMR detection of dipolar-coupled pairs of labels. The presence of an oxygenated β carbon is inferred from chemical-shift data. The structure of R is unknown.

N-β-alanyldopamine form covalent bonds with protein or chitin or both.

The ^{13}C and ^{15}N REDOR results for the β-alanine-labeled exuviae support the notion that *N*-β-alanyldopamine is acting as a versatile cross-linking agent in cuticle. The presence of unscrambled ^{13}C–^{15}N pairs indicates direct incorporation of β-alanyl fragments as illustrated by the scheme of Figure 5. The broad range of chemical shifts observed for the labeled β-alanyl nitrogen indicates the formation of new covalent bonds at the terminal nitrogen of *N*-β-alanyldopamine, including secondary amines which may be involved in cross-links. Further experiments are in progress to identify the carbons involved in these putative cross-links.

It is possible that some ^{13}C and ^{15}N double labels from β-alanine are incorporated in *Manduca sexta* via prior metabolism to glycine. However, radiolabel-tracer studies involving *Musca domestica* indicate that C-1 catabolism of β-alanine represents only a minor pathway in insect tissue.[22] In addition, we have observed that [2-^{13}C,^{15}N]glycine is incorporated into *Manduca* adult and pupal cuticles to a much lesser extent than double-labeled β-alanine and that all the ^{15}N label from double-labeled glycine is present in peptide bonds (data not shown). Thus, metabolism of β-alanine to glycine does not account for the functionalization of the β-alanyl nitrogen shown in Figure 4 (top right).

Proposal for Cross-Links in Insect Cuticle. The NMR results on chemical functionality in cuticle, as well as the documented chemical stability of sclerotized insect cuticle,[6-8] can be rationalized by the kind of structure shown in Figure 6. The central assumption of this structure is that oxidation products of catecholamines serve as cross-linking agents. Proteins may be cross-linked to other proteins or chitin through N-acylated dopamines in a variety of ways, making stabilized cuticle structurally heterogeneous. Some of the chemical features suggested in Figure 6 are clearly supported by the experimental NMR evidence that has already been presented, while others, particularly the linkage to chitin, are speculative. Additional REDOR experiments are in progress to identify covalent links to chitin and to the amino group of *N*-β-alanyldopamine.

Conclusions

The results of solids NMR experiments on labeled *M. sexta* pupal cuticle emphasize the central role of *N*-β-alanyldopamine in cuticle biochemistry. Two kinds of covalent bond formation of histidyl residues to *N*-β-alanyldopamine have now been established unambiguously by NMR spectroscopy: one to dopamine ring carbons[9] (by double-cross-polarization) and one to dopamine β carbons (by REDOR). Oxygen-substituted β carbons of *N*-β-alanyldopamine are also present. A fourth modification of *N*-β-alanyldopamine involves substitution reactions at the primary amino group derived from β-alanine. To our knowledge, this is the first observation of such modifications to the amino group of β-alanine in insect cuticle. Solid-state NMR analysis has now provided direct evidence of covalent modifications to the ring carbons, the β-carbon, and the terminal nitrogen of *N*-β-alanyldopamine in the pupal cuticle of *M. sexta*.

Acknowledgment. D,L-[*ring*-$^{15}N_2$]histidine was a gift from the Los Alamos Stable Isotope Resource. This work was supported by NSF Grants DIR-8720089, DIR-8714035, and DCB-86-09717 and by USDA Grant 88-CRCR-3684.

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