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STUDIES OF THE SCLEROTIZATION OF INSECT CUTICLE

THE STRUCTURE OF A DIMERIC PRODUCT FORMED BY INCUBATION OF *N*-ACETYLDOPAMINE WITH LOCUST CUTICLE

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Abstract—When locust cuticle is incubated together with N-acetyldopamine under aerobic conditions a compound is formed which is identical to one isolated in small amounts from naturally sclerotized cuticles. The structure has been determined by mass spectrometry and NMR spectroscopy. It is 2-(3',4'-dihydroxyphenyl)-3-acetylamino-6 (or 7)-(N-acetyl-2"-aminoethyl)-2,3-dihydro-1,4-benzodioxin.

Some years ago one of us isolated a compound which is formed when N-acetyldopamine (I) is incubated with locust cuticle. The compound was partly characterized and a structure II was suggested.[1] The structure cannot account for all the observed properties, and since the compound is important for understanding the chemical reactions occurring during sclerotization of insect cuticle, we decided to establish the structure by more unambiguous methods. On the basis of mass spectrometry and ^1H and ^{13}C NMR spectroscopy we can now propose a revised structure, 2-(3',4'-dihydroxyphenyl)-3-acetylamino-6-(or 7)-(N-acetyl-2"-aminoethyl)-2,3-dihydro-1,4-benzodioxin (IIIa and b).

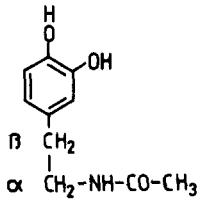
RESULTS AND DISCUSSION

The compound is readily formed when pieces of locust cuticle are incubated with N-acetyldopamine in the presence of oxygen. Anaerobic conditions or reducing agents, such as ascorbic acid, will block its formation, and neither is the compound formed, if cuticle is omitted or it it has been heated previously to 100° for 5 min. The compound can be purified by fractionation of the incubation medium on columns of BioGel P-2 and Sephadex LH-20. The compound is easily hydrolysed by acids, and the nature of hydrolysis products depends upon the conditions of hydrolysis. Reflux in 1 M HCl for 3 hr gives equimolar amounts of dopamine and hydroxyketocatechol (2-hydroxy-3',4'-dihydroxyacetophenone, IV), whereas hydrolysis in 6 M HCl at 110° for 3 hr gives dopamine together with both arterenone (V) and hydroxyketocatechol.[1] When the compound is incubated in conc HCl at 40° for 1 hr, the main products are N-acetyldopamine and N-acetylarterenone plus smaller amounts of the above-mentioned products.

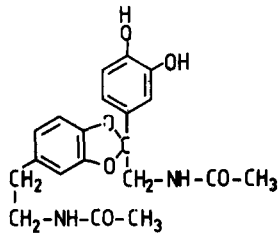
If the compound is methylated by means of diazomethane before hydrolysis, the ketocatechol fragment is recovered in its O-dimethylated form, and the dopamine fragment is unmethylated.[1] This indicates that the two phenolic groups in the dopamine part of the intact compound are involved in connecting it to the ketocatechol part, and that the compound accordingly may be a dimer of N-acetyldopamine. These observations led to the suggestion of structure II,[1] but it cannot explain that hydrolysis in dilute acid gives the alcohol (IV) in nearly quantitative yield, so the compound was subjected to mass spectrometry.

The e.i. mass spectrum of the compound is shown in Fig. 1. The peak at *m/e* 386 being the molecular peak was confirmed by the presence of peaks corresponding to losses of neutral molecules or radicals from *m/e* 386, thus, loss of ketene (*m/e* 344), MeCO· (*m/e* 343), $MeCONH_2$ (*m/e* 327), and $\cdot CH_2NH{-}CO{-}Me$ (*m/e* 314), and further by the field desorption (f.d.) mass spectrum which, at an emitter current of 18 mA only contained a peak at *m/e* 387, corresponding to M + 1. At increased emitter current two peaks at *m/e* 194 and 196 appeared. This may indicate thermal decomposition of the sample. Field ionization (f.i.) gave rise to peaks at *m/e* 386(10), 237(25), 235(30), 195(80), and 193(100). The relative intensities are given in brackets. The mass of the molecular peak was 386.1483 and the elementary composition; $C_{20}H_{22}O_6N_2$ (386.1478). The suggested fragment ions are given in Fig. 1. The composition of the fragment ions has been supported

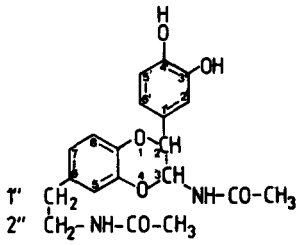
I



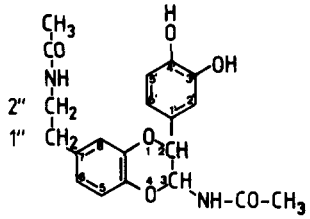
II



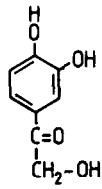
III a



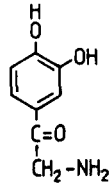
III b



IV



V



by exact mass determinations, metastable ion analysis, and comparison with the mass spectra of the below-mentioned derivatives of the compound.

Upon methylation with diazomethane two Me groups were introduced. The fragmentation patterns of methylated and underivatised products were rather similar, but the peaks corresponding to *m/e* 195, 235, and 237 in Fig 1 were not obtained with the methylated sample. The f.i. data indicated that these peaks are the result of thermal decomposition of the underivatized sample during evaporation. The fragment ions at *m/e* 235 and 237 have elementary compositions $C_{12}H_{13}N_1O_4$ and $C_{12}H_{15}N_1O_4$, respectively. Mass spectrometry of the *in vitro* synthesized dimer containing two trideutero-acetyl groups demonstrated that these ions were formed by acetyl transfer to the fragments at *m/e* 193 and 195.

The mass spectra agree with the suggested structure III. The main fragmentation is cleavage of the 1,4-dioxin ring resulting in the fragment ion correspond-

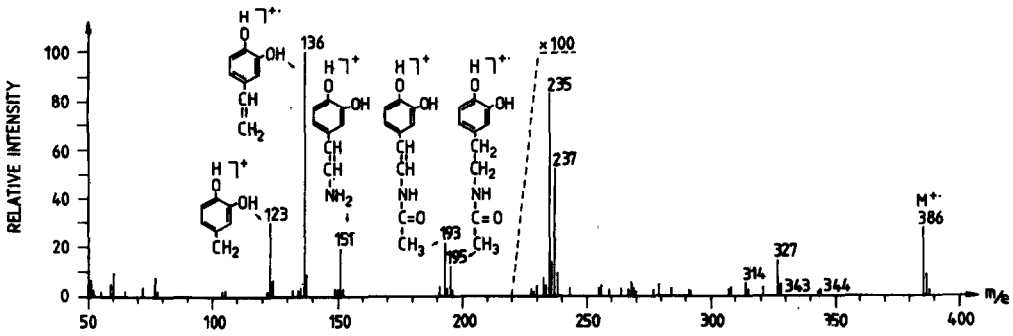


Fig. 1. Mass spectrum of the underivatised compound.

ing to *m/e* 193. This is in accordance with the data obtained for 1,4-dioxan which, upon electron impact ionization, as main fragment ion shows ethylene.[2,3]

An alternative structure containing a single oxygen bridge between the two N-acetyldopamine molecules and a double bond between two of the sidechain C atoms thus leaving a total of three free phenolic OH groups is also possible according to the mass spectrometrical data. This structure can be supported by the results obtained by acetylation which results in triacetylation with traces of tetraacetylation as judged by mass spectrometry. Two of the acetyl groups were O-acetyl groups on the dihydroxyphenyl ring. The position of the third (and fourth) acetyl group could not be deduced from the mass spectra due to extensive losses of ketene. This alternative structure is neither in agreement with the data obtained from hydrolysis of the compound nor with the methylation results.

The mass spectrometrical investigations thus demonstrate that the compound is composed of two N-acetyldopamine moieties, but the structure cannot be established unequivocally by this method. Therefore, sufficient material was synthesised for ^1H and ^{13}C NMR studies.

The ^1H NMR spectrum of the compound in methanol, d_4, shows the existence of two different Me groups giving rise to two separate lines at $\delta = 1.90$ ppm and $\delta = 1.87$ ppm. The signals from H(2) and H(3) in methanol, d_4, are composed by two doublets located at $\delta(H(2)) = 4.8$ ppm and $\delta(H(3)) = 5.8$ ppm, respectively. In acetone, d_6, and dimethylformamide, d_7, the signals from H(3) were split into a doublet by a coupling to the amido proton. The protons in the methylene groups give rise to two triplets at $\delta(H(1'')) = 2.7$ ppm and $\delta(H(2'')) = 3.4$ ppm. A broad unresolved line was observed from the aromatic protons.

Because of deuteration the signals from the NH and OH protons were unobserved in methanol, d_4 solution. By using acetone, d_6, or dimethylformamide, d_7, as solvents the signals from the exchangeable protons were observed. The OH protons could only be assigned to a very broad line at approximate $\delta = 14$ ppm. The amido proton coupled to H(3) was observed as a relatively sharp dublet at $\delta = 8.2$ ppm, while the other amido proton gave a broad line at 8.8 ppm.

The ^{13}C NMR spectrum of the compound in methanol, d_4, shows only one signal at $\delta = 22.61$ ppm from the Me groups due to coincidental equivalence. The two methylene C atoms resonate at $\delta(C(1'')) = 35.8$ ppm and $\delta(C(2'')) = 42.2$ ppm. The lines originating from the methine C atoms have tentatively been assigned to $\delta(C(2)) = 78.3$ ppm and $\delta(C(3)) = 120.7$ ppm. Sixteen lines from the aromatic C atoms are located in the region from 115 ppm to 148 ppm.

In order to explain the number of lines in the aromatic region it is necessary to assume the presence of two stereoisomers of either IIIa or IIIb. These two stereoisomers may arise since the two protons H(2) and H(3) in the dioxin ring system may be situated *cis* or *trans* with respect to each other. Such two stereoisomers would give different values of the chemical shift of the C atoms near the dioxin ring. The existence of two stereoisomers can also be seen from the ^1H NMR spectrum in acetone, d_6 solution. The signals from H(2) and H(3) are doubled in this spectrum, giving rise to a rather complicated spectrum in case of H(3) and four distinct lines in case of H(2). Contrary to the spectrum obtained in methanol, d_4 solution, the spectrum obtained in acetone, d_6 solution exhibits a difference in chemical shift of H(2) and H(3) between the two stereoisomers. This is mainly caused by a much smaller linewidth in the latter solution.

The carbonyl C atoms should in principle give two separate lines in the NMR spectrum, but in most solvents these two lines collapse to only one line. In dimethyl formamide, d_7, the difference in chemical shift is so pronounced that two signals can be observed at $\delta = 170.2$ ppm and 170.0 ppm, respectively.

The ^1H NMR spectra of the acetylated derivative of III are very similar to the spectra of the underivatized compound. Two extra lines due presumably to Me groups occur together with changes in the aromatic region. This implies that the dioxin ring system and the sidechain on the benzo ring are unchanged by acetylation. The two extra lines can be assigned to two O-acetyl groups and an acetyl group bonded neither to oxygen nor nitrogen, but attached for example to an aromatic ring. The carbon spectrum shows that the compound most likely contained more than one derivative. A complete assignment of the spectrum was therefore impossible. Acetylation of the aromatic ring due to a Friedel–Craft type of reaction could by no means be excluded, and occurrence of such a reaction would in fact explain the appearance of the NMR spectra.

The results obtained by NMR thus clearly show that structure IIIa or b are the only structures that can account for the observed spectra, and structure II as well as the alternative structure, which is possible according to the mass spectra, are incompatible with the NMR spectra.

Structure III agrees with results obtained by using N-acetyldopamine tritiated on either the α- or β-carbon atom for dimer-formation. With both tritiated substrates part of the radioactivity was released as tritiated water, and the distribution of radioactivity in the purified dimer corresponded to removal of one hydrogen from both the α- and β-position. Structure III can also explain the finding that acid hydrolysis can result in either an amino or a OH group in the ketocatechol which is formed, since both an O and a N atom are connected to C atom C(3) in the dimer.

The aim of this investigation was to establish the nature of the bonds involved in forming the dimer. Therefore, we have not attempted to distinguish between the possible isomers IIIa and IIIb. Neither have we attempted to resolve the possible *cis-trans* isomerism and the stereochemical configuration of the molecule. However, during purification of the dimer a minor component was also obtained. This component was identical to the major component in all respects which were investigated: UV spectrum, mass spectra and hydrolysis behaviour. It can be assumed that one of the components is IIIa and the other IIIb.

The dimer is formed during natural hardening of the cuticle. The natural material will also separate in a minor and a major component during purification, and the mass spectra and the NMR spectra of the major *in vivo* component are identical to those of the *in vitro* compound. One or more enzymes in the cuticle are responsible for the formation of the compound.[4] We do not know what intermediates are involved in

the formation, but from the structure of the compound it is likely that an unsaturated derivative of N-acetyldopamine could be involved. In that case, sclerotization of insect cuticle will have similarities to the formation of lignin in plants from coniferyl alcohol.

Only a few percent of the N-acetyldopamine incorporated into the cuticle can be recovered as dimers. The majority appears to be linked to the cuticular proteins by linkages of unknown nature. Since these protein-connected N-acetyldopamine-residues also are liberated as ketocatechols on acid hydrolysis, and since there is the same relationship between acid strength and type of ketocatechols obtained for the cuticular-bound N-acetyldopamine as for the dimer, it appears reasonable to assume that N-acetyldopamine in the cuticle is bound to the proteins through linkages going from the two C atoms in the side-chain of N-acetyldopamine to perhaps phenolic groups or amino groups in the proteins. Protein-linked N-acetyldopamine containing free phenolic groups may also provide binding sites for additional N-acetyldopamine-residues resulting in the formation of protein-bound dimers and higher polymers.

EXPERIMENTAL

Synthesis and purification of dimers. Manually cleaned cuticle from femurs of about one hundred locusts (*Locusta migratoria*) was incubated overnight at room temp in 100 ml 0.2 M Na-acetate buffer, pH 5.5, containing 100 mg Na_2EDTA and 200 mg N-acetyldopamine. After the incubation period the supernatant was concentrated and fractionated on a column of Bio-Gel P-2 (5 cm^2 × 90 cm). Elution was performed with 0.2 M AcOH and the fractions, which according to UV spectra contained dimers, were pooled and refractionated on a column of Sephadex LH-20 (2 cm^2 × 15 cm) with 0.2 M AcOH as eluant.

Mass spectrometry. The mass spectra were recorded on a Varian Mat 311 A Mass Spectrometer (Danish Science Research Council Grant No. 511-3809) equipped with an e.i. or a combined e.i., f.i., f.d. ion source. The e.i. conditions were: ion source temp 210–250°, ionizing voltage 70 eV. The sample was introduced directly into the ion source in a quartz crucible. The direct inlet temp was 180–200° for derivatised compound and 260–280° for underivatised compound.

Exact mass determinations were carried out with an accuracy better than 10 ppm by peak matching. Metastable analysis was carried out using the facilities for scanning either with the accelerating voltage or with the electrostatic sector voltage of the 311 mass spectrometer. The f.d. spectra were obtained using benzonitrile activated emitters. The sample was applied on the emitter from methanolic solution by the dipping technique.

Derivatisation. Methylation was carried out by reacting the compound dissolved in MeOH with diazomethane in ether for 24 hr at room temp, followed by evaporation of solvents and reagent with a N_2 stream.

Acetylation was carried out by reacting 10–20 fold excess of Ac_2O for 2 hr at 60° with the compound dissolved in pyridine p.a., followed by removal of the solvent and reagent under vacuum.

NMR spectroscopy. The ^1H and ^{13}C NMR spectra were recorded on a Jeol FX60Q FT NMR spectrometer. The magnetic field was stabilized by internal deuterium lock. The spectral width was 1000 Hz for proton and 4000 Hz for carbon spectra. 8K data points were used. All chemical shifts were measured as δ (ppm-downfield to TMS). The assignments of the lines in the noise decoupled ^{13}C NMR spectrum were partly performed by extensive use of the non decoupled and selective decoupled carbon spectra.[5] Careful use of homo decoupling experiments has been a great help in clarifying the coupling patterns in the ^1H NMR spectrum.

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