5-Acetyl-2,3-dihydro-1*H*-pyrrolizines and 5,6,7,8-Tetrahydroindilizin-8-ones, Odor Constituents Formed on Heating L-Proline with D-Glucose

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The reaction products of the equimolar mixture of L-proline and D-glucose heated at 200° for 6 min were examined. From the ethyl acetate extract of the reaction products, new nitrogenous compounds, 5-acetyl-2,3-dihydro-1*H*-pyrrolizine, 5-acetyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine, 5-formyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine, and 5,6,7,8-tetrahydroindilizin-8-one and its methyl derivative, were isolated and identified. In addition to the above compounds, the following were identified: 2-furfuryl alcohol, quinoline, 3,5-dihydroxy-2-methyl-4*H*-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one, 2-hydroxy-3-methyl-2-cyclopenten-1-one, and 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone. Isolation and identification were based on preparative gas chromatography and spectroscopic methods (mass, ir, uv, and nmr spectra). The nitrogenous compounds had a mild smoky, weak amine-like odor with a somewhat bitter burnt tone. The caramel and burnt sugar fragrance of 2-hydroxy-3-methyl-2-cyclopenten-1-one and 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone seemed to be modified slightly toward roasted, bread-like nuance by the trace addition of these nitrogenous compounds.

It has been demonstrated by many workers (Hodge, 1953, 1967; Mason and Waller, 1964; Newell *et al.*, 1967; Pinto and Chichester, 1966; Rohan and Stewart, 1966; Rohan, 1970) that the Maillard reaction contributes to the flavors and aromas of the roasted and cooked foods as well as the development of the brown color. Many aroma substances have been obtained from the model system of this reaction (Hodge *et al.*, 1963; Kato, 1966, 1967; Kato and Fujimaki, 1970; Koehler *et al.*, 1969; Mills *et al.*, 1969, 1970a; Rizzi, 1969; Shigematsu *et al.*, 1971).

The reactions of L-proline, L-hydroxyproline, pyrrolidine, and piperidine with D-glucose, glycerol, and dihydroxyacetone produce the bread and cracker-like aroma. 1-Azabicyclo[3.3.0]oct-4-one, 1-methyl-2-acetylpyrrolidine, 1,4,5,6-tetrahydro-2-acetylpyridine (Hunter *et al.*, 1966a,b, 1969), 1-acetonylpyrrole, and 1-acetonylpyrroline (Kobayashi and Fujimaki, 1965) were identified as the aroma substances from the above reaction system. 2,3-Dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one was identified as the main product from the thermal degradation of 1-deoxy-1-L-prolino-D-fructose (Mills *et al.*, 1970b). L-Proline and 1-deoxy-1-L-prolino-D-fructose were also reported to be main components among the amino acids and the Amadori compounds in flue-cured tobacco leaf (Tomita *et al.*, 1965) and it has been generally recognized that the most abundant sugar in flue-cured tobacco leaf is D-glucose. These results suggest that the reaction of L-proline with D-glucose and the thermal degradation of 1-deoxy-1-L-prolino-D-fructose might have an important role in the formation of the flavor and aroma of tobacco smoke as well as those of the roasted and cooked foods.

In connection with these respects, further investigation of the compounds produced by the reaction of L-proline with D-glucose was undertaken. In the present study, L-proline and D-glucose were allowed to react at 200° for 6 min and the reaction products were examined. As a result several new odor compounds, 5-acetyl-2,3-dihydro-1*H*-pyrrolizine, 5-acetyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine, 5-formyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine, and 5,6,7,8-tetrahydroindolizin-8-one and its methyl derivative, were identified in addition to the known sugar–amino acid reaction products.

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EXPERIMENTAL SECTION

Melting points were uncorrected. The mass spectra were recorded with a Hitachi RMU-7 mass spectrometer. The ir spectra were obtained with a JASCO IR-G spectrophotometer. The nmr spectra were obtained with a JNM-PS-100 nmr spectrometer in deuteriochloroform with tetramethylsilane as an internal standard. The uv spectra were run on a Hitachi recording spectrophotometer Model EPS-3T.

Sample Preparation. One-tenth mole of L-proline and 0.1 mol of D-glucose were mixed thoroughly, and the mixture was put into a round-bottomed flask which had been preheated at 200° and heated at the same temperature for 6 min. After cooling below 100°, the reaction mixture was dissolved in 100 ml of water. The resultant solution was extracted three times, once with 200 ml of ethyl acetate and twice with 100 ml of ethyl acetate. The extracts were combined and washed with saturated sodium chloride aqueous solution, dried with anhydrous sodium sulfate, and concentrated *in vacuo*. These procedures were repeated 70 times to obtain a dark brown extract (43.5 g).

Fractionation of the Ethyl Acetate Extract. Fractionation of the ethyl acetate extract to the acidic, basic, and neutral fractions was carried out by the conventional procedure using 5% sodium bicarbonate and 5% hydrogen chloride aqueous solutions to afford 2.7 g of the acidic, 17.3 g of the basic, and 4.8 g of the neutral fractions. In the present investigation, the neutral and basic fractions were used for the identification of the components.

Column Chromatography. Separation of the basic and neutral fractions by silicic acid column chromatography is summarized in Table I. The neutral concentrate was mounted on a silicic acid column. The column was developed successively with benzene, benzene–ethyl acetate mixture, and finally methanol. The eluates were separated into ten fractions. The basic fraction was fractionated in the same manner to give 11 fractions as shown in Table I.

Gas Chromatographic Methods. For collection of the compounds, a Hitachi K-53 gas chromatograph equipped with a dual thermal conductivity detector was used. Samples were injected onto a 3 m × 3 mm i.d. stainless steel column packed with 20% Carbowax 20M or 20% Apiezon grease L on 60–80 mesh Chromosorb W. Effluent fractions were collected in a glass capillary tube cooled with a Dry Ice–methanol bath. Repeated collections were made with a Carbowax 20M column until sufficient quantities were obtained. The contents of the glass capillary tube were further fractionated with an Apiezon grease L column.

Table I. Separation of Basic and Neutral Fractions by Column Chromatography

Compd no.	Eluate	Yield, g	Aroma
	Basic Fraction[a]		
1	Benzene	0.75	Sweet caramel
2	10% EtOAc–benzene	1.04	Caramel, nutty
3	15% EtOAc–benzene	0.84	Smoky, sweet, amine-like
4	20% EtOAc–benzene	1.55	
5	20% EtOAc–benzene	1.28	
6	40% EtOAc–benzene	1.04	
7	50% EtOAc–benzene	1.03	
8	70% EtOAc–benzene	0.88	
9	90% EtOAc–benzene	0.67	
10	Ethyl acetate	1.04	
11	Methanol	2.10	
	Neutral Fraction[b]		
1	Benzene	0.33	Sweet, smoky, weak,bread-like
2	5% EtOAc–benzene	0.28	Sweet, smoky, weak bread-like
3	10% EtOAc–benzene	0.25	Caramel, weak smoky
4	10% EtOAc–benzene	0.45	Caramel, burnt sugar like
5	15% EtOAc–benzene	0.35	Caramel, burnt sugar like
6	20% EtOAc–benzene	0.25	Caramel, burnt sugar like
7	30% EtOAc–benzene	0.39	Sweet, weak caramel
8	40% EtOAc–benzene	0.25	
9	60% EtOAc–benzene	0.28	
10	Methanol	0.27	

[a] Column: Mallinckrodt, 400 g, 5 cm × 35 cm. [b] Column: Mallinckrodt, 125 g, 4 cm × 30 cm.

Table II. Compounds Identified in Basic and Neutral Fractions

No.	Compound	Fraction no.	RT,[a] min	Aroma
	Basic Fraction			
1	2-Hydroxy-3-methyl-2-cyclopenten-1-one	1	22.8	Caramel, maple-like
2	3,5-Dihydroxy-2-methyl-4*H*-pyran-4-one	2, 3	34.4	
3	Quinoline	3	26.8	Pyridine-like
4	2,3-Dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one	4, 5	32.9	
	Neutral Fraction			
5	5-Acetyl-2,3-dihydro-1*H*-pyrrolizine	1	28.4	Smoky, sweet,weak amine-like
6	5-Formyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine	1	31.9	Smoky, sweet, cinnamic
7	5-Acetyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine	1	32.4	Smoky, bitter, medicine-like
8	2-Furfuryl alcohol	2	19.6	Burnt
9	5, 6, 7, 8-Tetrahydroindolizin-8-one	3	36.4	Mild smoky, weak amine-like
10	2-Methyl-5,6,7,8-tetrahydroindolizin-8-one	3	39.0	Mild smoky, weak amine-like
11	2,5-Dimethyl-4-hydroxy-3(2*H*)-furanone	4–6	28.0	Caramel, burnt sugar-like
2	3,5-Dihydroxy-2-methyl-4*H*-pyran-4-one	4–6	34.4	

[a] Retention time on Carbowax 20M column.

The temperature of both columns was elevated from 80 to 250° at a rate of 5°/min and kept at 250°. Helium was used as a carrier gas at a flow rate of 60 ml/min at 80°. The injection temperature was 230° and the detector temperature was 250°. For the purification of the synthesized 5-acetyl-2,3-dihydro-1*H*-pyrrolizine and 5-formyl-2,3-dihydro-1*H*-pyrrolizine, preparative gas chromatography was performed. A Hitachi F6-D gas chromatograph modified for preparative use, with a 3 m × 3 mm i.d. stainless steel tube packed with 20% Carbowax 20M on 60–80 mesh Chromosorb W, was used. The column temperature was maintained at 200°. The carrier gas was helium at a flow rate of 40 ml/min. The injection and detector temperatures were the same as those described above.

Authentic Sample. 2-Hydroxy-3-methyl-2-cyclopenten-1-one, 2-furfuryl alcohol, and quinoline were obtained commercially. 2,5-Dimethyl-4-hydroxy-3(2*H*)-furanone was obtained as previously reported (Kato *et al.*, 1972).

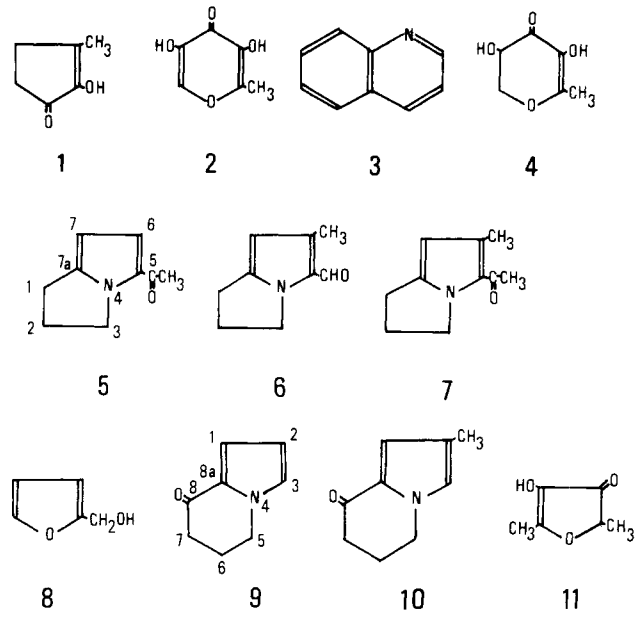
5-Acetyl-2,3-dihydro-1*H*-pyrrolizine. 2,3-Dihydro-1*H*-pyrrolizine was prepared by Wolf–Kischner reduction of 2,3-dihydro-1*H*-pyrrolizin-1-one obtained by the methods described by Meinwald and Meinwald (1966). To a solution of 2.8 g of freshly distilled 2,3-dihydro-1*H*-pyrrolizine in 6.5 ml of anhydrous ether was added 2.6 ml of magnesium bromide in ether (Swain and Boyles, 1951), and the mixture was cooled in an ice bath. To the stirred mixture was added dropwise 2.5 g of acetyl chloride in 5.2 ml of anhydrous ether. After decomposition of the reaction product with ice water, the solution was extracted with ether. The ether extract was washed with 5% sodium carbonate aqueous solution, dried over anhydrous sodium sulfate, and concentrated *in vacuo*. The concentrate was chromatographed on a silica gel column (Mallinckrodt, 10 g, 2 × 10 cm) using first benzene and then 5% ethyl acetate in benzene as eluents. The fractions eluted with 5% ethyl acetate in benzene were collected and concentrated *in vacuo* to give 0.91 g of oily concentrate. A part of the concentrate was fractionated by preparative gas chromatography. The collection of the main fraction which appeared at a retention time of 10 min gave colorless needles of 5-acetyl-2,3-dihydro-1*H*-pyrrolizine: mp 45.5°; λ_{max} (EtOH) 296 nm (ϵ 1.66 × 10^4). *Anal.* Calcd for $C_9H_{11}ON$: C, 72.45; H, 7.43; N, 9.39. Found: C, 72.75; H, 7.41; N, 9.42.

5-Formyl-2,3-dihydro-1*H*-pyrrolizine. To 2.8 g of dimethylformamide, which was contained in a 50-ml, three-necked flask cooled with an ice bath, was added 1.6 ml of phosphorus oxychloride with stirring over a period of 10 min. To the mixture was added 2 ml of ethylene dichloride. When the solution had cooled to 5°, 1.5 g of freshly distilled 2,3-dihydro-1*H*-pyrrolizine in 6 ml of ethylene dichloride was added to the stirred, cooled mixture over a period of 1 hr at such rate that the temperature remained below 20°. The stirred mixture was then refluxed for 15 min and cooled to room temperature. A solution of 11.3 g of sodium acetate trihydrate in 16 ml of water was added to the stirred mixture and the resultant mixture was re-

Table III. Mass Spectra of Identified Compounds

Compd	*m/e* (relative abundance, %)
1	112 (100), 97 (8), 84 (14), 83 (19), 69 (38), 56 (16), 55 (27), 43 (26)
2	142 (82), 113 (20), 101 (27), 85 (21), 68 (30), 55 (33), 44 (45), 43 (100)
3	129 (100), 128 (19), 102 (36), 76 (18), 75 (16), 51 (22), 50 (19)
4	144 (54), 115 (9), 101 (44), 73 (29), 72 (27), 55 (23), 45 (25), 44 (70), 43 (100)
5	149 (96), 134 (100), 106 (22), 79 (19), 78 (13), 77 (17), 52 (11), 51 (15), 43 (20)
6	149 (96), 148 (100), 134 (32), 120 (77), 106 (18), 93 (29), 77 (30), 66 (40)
7	163 (30), 149 (32), 148 (100), 134 (10), 120 (31), 93 (13), 65 (26)
9	135 (100), 107 (33), 106 (49), 80 (25), 79 (73), 52 (30), 43 (17), 39 (31)
10	149 (50), 121 (20), 120 (23), 106 (23), 93 (100), 79 (17), 66 (23), 53 (22)
11	128 (57), 85 (24), 55 (17), 43 (100)

Chart I. Structures of Identified Compounds



fluxed for 15 min and cooled to room temperature. The organic phase was separated and washed with a saturated sodium carbonate aqueous solution, dried over anhydrous sodium sulfate, and concentrated *in vacuo*. The concentrate was chromatographed on a silicic acid column in the same manner as described above. The fractions eluted with 5% ethyl acetate in benzene were collected and concentrated *in vacuo* to give 0.58 g of an oily concentrate which was then fractionated by preparative gas chromatography. The colorless oil of 5-formyl-2,3-dihydro-1*H*-pyrrolizine was obtained by collection of the main fraction which appeared at the retention time of 9.7 min; λ_{max} (EtOH) 296 nm (ϵ 9.61 × 10^3). *Anal.* Calcd for C_8H_9ON: C, 71.09; H, 6.71; N, 10.36. Found: C, 70.43; H, 6.62; N, 10.19.

5,6,7,8-Tetrahydroindolizin-8-one was prepared as described by Clemo and Ramage (1931).

RESULTS AND DISCUSSION

It is generally known that, even in the reaction of the same amino acid with D-glucose, the aroma varies with the reaction temperature and with reaction time. Initially, the equimolar mixture (0.1 mol) of L-proline and D-glucose was constantly heated at 200° and the aroma developed during the reaction was evaluated continuously. The aroma developed during the first 5 min was weak cake-like; the pleasant caramel and burnt sugar-like aroma with a roasted bread-like tone became intense during the next 3 min, and an undesirable odor became predominant thereafter. Therefore, our attention was focused on the reaction products yielded by heating at 200° for 6 min, which was the reaction condition in the present study.

In Table II are listed the compounds identified in the basic and neutral fractions, together with the fraction number, retention time on Carbowax 20M column, and characterization of aroma. The mass spectral data are shown in Table III and the structures of the identified compounds are shown in Chart I. The acidic fraction had a strong acetic acid like odor and was poor in caramel, burnt sugar, and bread-like nuances. So, this fraction was not investigated in the present study. As shown in Tables I and II, aroma compounds were eluted by column chromatography in the early part of the effluent.

Compounds 1, 3, 8, and 11 were identified by interpretation of their mass and ir spectra and confirmation was made by comparison of their spectral data with those of the authentic samples. Compound 2 was identified as 3,5-dihydroxy-2-methyl-4*H*-pyran-4-one by comparison of its ir and mass spectra with those reported by Terada *et al.* (1962) and Jurch and Tatum (1970). Shaw *et al.* (1971) reported the formation of this compound by oxidation of 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one. Compound 4 was identified as 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one by comparison of its ir and mass spectral data with those reported by Mills *et al.* (1970b). This compound has been isolated from stored dehydrated orange juice (Tatum *et al.*, 1967), the degradation products of D-fructose (Shaw *et al.*, 1967, 1968), and the degradation products of D-glucose with methylamine and acetic acid (Jurch and Tatum, 1970).

Compounds 2 and 4 were the major products contained in the ethyl acetate extract. Compound 2 was the major component in the neutral fractions 4–6 and the basic fractions 2 and 3. The main component in the basic fractions 4 and 5 was compound 4. Although these compounds were present in large quantity, their contribution to aroma appeared to be insignificant. Compounds 2 and 4 were odorless in a pure state at room temperature. Hodge *et al.* (1972) reported that compound 4 might be formed *via* 2,3 enolization of 1-deoxy-1-L-prolino-D-fructose and the methyl-α-dicarbonyl intermediate and postulated that 2-methyl-3-hydroxy-4*H*-pyran-4-one (maltol) might be produced by dehydration of 2,3-dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one (4). Compound 2 would be produced by further oxidation of 4.

Compounds 1 (2-hydroxy-3-methyl-2-cyclopenten-1-one) and 11 (2,5-dimethyl-4-hydroxy-3(2*H*)-furanone) have very pleasant caramel and burnt sugar-like aromas. They are considered to be the key components contributing to the caramel and burnt sugar-like fragrances developed by the reaction of L-proline with D-glucose. Quinoline (3) has a characteristic pyridine or amine-like odor and probably provides smoky and tar-like tones to a sweet caramel-like aroma.

Five new nitrogenous compounds were isolated from the neutral fraction. Their ir and uv spectral data are shown in Table IV. Compound 5 had a strong ir absorption at 1637 cm^{-1} suggestive of a carbonyl group. The uv spectrum showed an absorption maximum at 296 nm with a shoulder around 250 nm. These spectral data were found to be similar to those of pyrrole compounds with a carbonyl group, as reported in previous papers (Kato and Fujimaki, 1970, 1972; Shigematsu *et al.*, 1971). The mass spectrum of 5 had its parent peak at *m/e* 149 correspond-

Table IV. Ir and Uv Spectra of Nitrogenous Compounds

Compd	Ir spectra, cm^{-1} [a]	Uv spectra in ethanol, nm	
		λ_{max}	Shoulder
5	1637 s, 1529 w, 1462, 1402, 1348, 1273, 1109, 1047, 944, 766	296	250
6	1630 s, 1526 w, 1485, 1460, 1430, 1417, 1383, 1369, 1348, 1283, 1264, 1065, 925, 892, 785	304	260
7	1625 s, 1530 w, 1488, 1455, 1434, 1406, 1375, 1340, 1288, 1096, 1057, 952, 929, 785	301	260
9	1645 s, 1524, 1480, 1456, 1412, 1386, 1345, 1246, 1196, 1164, 1080, 1067, 1034, 746	284	245
10	1645 s, 1556, 1484, 1457, 1392, 1373, 1239, 1194, 1162, 1125, 1006, 917, 862, 818	295	250

[a] s, strong; w, weak.

ing to the molecular formula $C_9H_{11}ON$. The major fragmentation peaks at *m/e* 134 (M − CH_3) and 106 (M − CH_3 − CO) suggested the presence of an acetyl group in the molecule. The nmr spectrum of **5** showed the presence of an acetyl group (δ 2.30, 3 H, s), two olefinic protons coupled to each other (δ 5.72, 1 H, d; δ 6.76, 1 H, d; J = ~4 Hz), indicative of two protons on a pyrrole ring. Two triplet signals at δ 2.76 (2 H) and 4.21 (2 H), and a multiplet signal at δ 2.46 (2 H) are indicative of the presence of $CH_2CH_2CH_2$ in a deshielded environment. Combining these structural features with those derived from the ir and uv spectral data leads to the structure of **5** as shown in Chart I. The position of the acetyl group was determined on the basis of the nmr spectrum. The coupling constant between C_6 and C_7 protons on the pyrrole ring is known to be *ca.* 3.5 Hz, while 5,6-coupling is *ca.* 2.5 Hz and 5,7-coupling is 1.5 Hz (White, 1963). The observed splitting of *ca.* 4 Hz in **5** indicates that the positions of two protons on the pyrrole ring are at C_6 and C_7. Accordingly, the position of the acetyl group is deduced to be at C_5.

The structure of **5** was confirmed as 5-acetyl-2,3-dihydro-1*H*-pyrrolizine by the coincidence of its spectral data with those of the synthetic specimen. Similarity of the ir and uv spectra of **6** and **7** to those of **5** suggested that the structures of **6** and **7** were analogous to that of **5**. The mass spectrum of **6** had the same parent peak at *m/e* 149 as **5**, but large differences were observed among the fragmentation peaks. The major peaks at *m/e* 148 (M − H) and 120 (M − CHO) suggested the presence of a formyl group attached to the pyrrole ring. The nmr spectrum of **6** showed the presence of a methyl group (δ 2.28, 3 H, s), one olefinic proton (δ 5.64, 1 H, s), a formyl proton (δ 9.59, 1 H, s), and $CH_2CH_2CH_2$ (δ 2.34, 2 H, m; δ 2.70, 2 H, t; δ 4.17, 2 H, t). These results suggested that **6** was a formyl methyl derivative of 2,3-dihydro-1*H*-pyrrolizine. The positions of the formyl and methyl groups were determined by comparison of its nmr spectrum with that of the synthetic 5-formyl-2,3-dihydro-1*H*-pyrrolizine. The nmr spectrum of the synthetic 5-formyl-2,3-dihydro-1*H*-pyrrolizine showed the following: δ 2.56, 2.82, 4.23 (2 H, m; 2 H, t; 2 H, t), 5.80, 6.71 (1 H, d; 1 H, d; J = *ca.* 4 Hz; C_6 and C_7 protons on a pyrrole ring, respectively), 9.22 (1 H, s, CHO). The appearance of one methyl signal with the disappearance of a signal corresponding to the C_6 proton in **6** indicated that the C_6 proton was replaced by the methyl group. Thus, it is deduced that **6** is 5-formyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine. Compound **7** showed its parent peak at *m/e* 163 in the mass spectrum. The major fragmentation peaks at *m/e* 148 and 120 suggested the presence of an acetyl group. The nmr spectrum of **7** showed signals at δ 2.28 (6 H, s, $COCH_3$, CH_3), 2.30, 2.70, 4.22 (2 H, m; 2 H, t; 2 H, t; $CH_2CH_2CH_2$), and 5.58 (1 H, s, C_7 proton on a pyrrole ring). From these results, the structure of **7** could be determined as 5-acetyl-6-methyl-2,3-dihydro-1*H*-pyrrolizine.

The ir and uv spectral data of **9** and **10** also suggested that these compounds had the carbonyl groups and pyrrole moieties in their molecules. The mass spectrum of **9** showed its parent ion at *m/e* 135 corresponding to the molecular formula of C_8H_9ON. The nmr spectrum showed the presence of $CH_2CH_2CH_2$ (δ 2.30, 2 H, m; δ 2.55, 2 H, t; δ 4.06, 2 H, t) and three olefinic protons coupled to each other (δ 6.16, 1 H, q; δ 6.6–6.7, 2 H, two q; three protons on a pyrrole ring). None of the signals corresponding to the formyl and acetyl groups were observed. These results lead to the keto-5,6,7,8-tetrahydroindolizine structure for **9**. Compound **9** was identified as 5,6,7,8-tetrahydroindolizin-8-one by the coincidence of its spectral data with those of the synthetic specimen. Compound **10** showed the parent ion at *m/e* 149 in the mass spectrum. Comparing the nmr spectrum with that of **9**, the appearance of a 3 H singlet at δ 2.06 and the changes in the olefinic region indicated that **10** was a methyl derivative of **9**. The poorly resolved two olefinic signals at δ 6.54 and 6.72 were supposed to be due to 1,3 protons coupling on the pyrrole ring. Thus, **10** was tentatively identified as 2-methyl-5,6,7,8-tetrahydroindolizin-8-one.

The odor of these nitrogenous compounds was mild, smoky, and weak amine-like with somewhat of a bitter tone. The caramel and burnt sugar fragrances of 2-hydroxy-3-methyl-2-cyclopenten-1-one and 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone appeared to be modified slightly toward a roasted, bread-like nuance by the trace addition of these nitrogenous compounds. The formation pathways of these nitrogenous compounds, which are reported for the first time in this work, are unknown at present.

ACKNOWLEDGMENT

The authors wish to express their thanks to Yoichi Kaburaki and Masao Noguchi of Japan Tobacco and Salt Public Corporation for their interest and encouragement during the course of this work.

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Received for review May 6, 1974. Accepted October 7, 1974.

A Chemical Assay for Saxitoxin, the Paralytic Shellfish Poison

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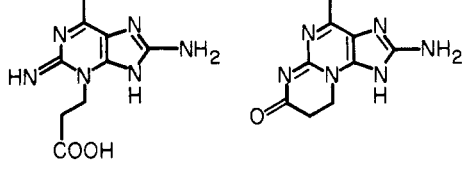
A chemical assay for saxitoxin, the paralytic shellfish poison, has been developed. The technique involves alkaline hydrogen peroxide oxidation of saxitoxin to 8-amino-6-hydroxymethyl-2-iminopurine-3(2*H*)-propionic acid (**1**), the fluorescence of which is measured at pH 5. This chemical assay is 100 times more sensitive than the existing bioassay and eliminates various problems associated with the bioassay, particularly at low levels of toxin. The method was developed initially with pure saxitoxin and has been applied to a number of samples of marine bivalves *Saxidomus giganteus* and *Mytilus californianus*.

Saxitoxin, which is one of the most toxic substances known, occurs in various bivalves endemic to the Pacific coast of America. The toxin originates in *Gonyaulax catenella* and is concentrated by shellfish which feed on these microscopic dinoflagellates (Schantz *et al.*, 1966). Human consumption of shellfish contaminated with saxitoxin causes poisoning and sometimes death; the human lethal dose is estimated to be ~1 mg, orally. This has necessitated the imposition of a permanent quarantine on clams in Alaska and on mussels from May through October in California.

Saxitoxin in shellfish is presently detected *via* mouse bioassay, which is sensitive to as little as 0.3 μg of saxitoxin per g of shellfish (Schantz *et al.*, 1958). In practice, however, this lower limit cannot be reached, since the presence of sodium ions in the shellfish counteracts the effect of saxitoxin. Several other problems are associated with the bioassay. (a) Before the concentration can be measured with any accuracy, several dilutions must be prepared and assayed. (b) A number of mice of the proper weight must be used for significant results, and there are differences in the susceptibility of different mouse strains. (c) The bioassay is not particularly sensitive and is not specific for saxitoxin.

An antigen–antibody assay has been attempted; however, the results were not promising (Johnson and Mulberry, 1966). A chemical assay utilizing a picrate complex has been reported (McFarren *et al.*, 1958) but it is nonspecific and less sensitive than the bioassay. Another procedure (Neve, 1972), in which saxitoxin is coupled with 1-fluoro-2,4-dinitrobenzene, appears to possess the same disadvantages.

The structure of saxitoxin was recently elucidated (Wong *et al.*, 1971b) and in the course of these studies, saxitoxin was degraded to 2-amino-8,9-dihydro-4-hydroxymethylpyrimido[2,1-*b*]purin-7(1*H*)-one (**2**) (Wong *et al.*, 1971a). Actually, the initial product of alkaline hydrogen peroxide oxidation of saxitoxin is 8-amino-6-hydroxymethyl-2-iminopurine-3(2*H*)-propionic acid (**1**) which forms **2** upon acid isolation. The structure of **1** suggested that it should be possible to design a chemical assay for saxitoxin based on the ultraviolet absorption or fluorescence of **1**.



1 2

EXPERIMENTAL SECTION

Saxitoxin dihydrochloride was isolated from *Saxidomus giganteus* by the standard procedure (Schuett and Rapoport, 1962) and stored at −13° in a sealed ampoule as an ethanol solution. Final purification of a standard for both chemical and bioassay was carried out as follows.

Saxitoxin (22 mg, 67% pure by bioassay) was dissolved in pH 5 sodium acetate buffer (0.2 *M*, 1.5 ml). The water was evaporated *in vacuo* and the saxitoxin redissolved in the same volume of water. This sample was then applied to a column (0.6 × 21 cm) of Bio-Rex 70, 50–100 mesh, H^+ form (prepared by careful washing, then elution with 1 *M* HCl followed by H_2O until the effluent pH was 5.0), and rinsed with 10 ml of H_2O. Saxitoxin was eluted with 0.05 *M* acetic acid (45–90 ml, 0.25 ml/min). The solvent was evaporated and the saxitoxin redissolved in aqueous HCl (pH 2) repeating this process three times. Finally, the dry saxitoxin was dissolved in H_2O and the pH was adjusted to 5 with AGl-X8, OH^- form. After filtering and evaporating the solvent, the saxitoxin was redissolved in a small volume of 85% EtOH, and purified by chromatography on acid-washed alumina. The ethanol solvent was removed by codistillation with water, and the saxitoxin was dried to constant weight to give an analytically pure sample (12 mg, 80% recovery) of saxitoxin dihydrochloride.

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