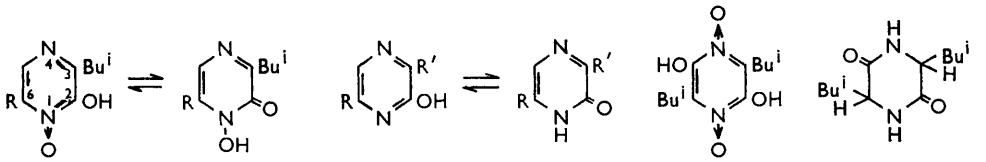
287. *Metabolites of* Aspergillus sclerotiorum *Huber.*

By R. G. Micetich and J. C. MacDonald.

Neohydroxyaspergillic acid and a compound isomeric with aspergillic acid were isolated from cultures of *Aspergillus sclerotiorum* Huber. The nuclear magnetic resonance spectra of these compounds, compared with those of various pyrazine derivatives, indicated that neohydroxyaspergillic acid was 2-hydroxy-6-(1-hydroxy-2-methylpropyl)-3-isobutylpyrazine 1-oxide (II), and that the other compound was 2-hydroxy-3,6-di-isobutylpyrazine 1-oxide (I). The latter compound has been reduced to the base (VI).

Various cyclic hydroxamic acids have been isolated from cultures of the *Aspergillus* species. These include aspergillic acid [1,2] (III), hydroxyaspergillic acid [3] (IV), and granegillin,[4] from *Aspergillus flavus*; muta-aspergillic acid [5,6] (V) from *Aspergillus oryzae*; and neohydroxyaspergillic acid [7] from *Aspergillus sclerotiorum*. Two other closely related compounds have been isolated; flavacol [8] (VI) from *Aspergillus flavus*, and pulcherriminic acid [9] (IX) from *Candida pulcherrima*.

In this study, two acidic compounds were isolated from *Aspergillus sclerotiorum*. Compound I, $C_{12}H_{20}N_2O_2$, had the same elemental analysis as aspergillic acid but had a different melting point and optical rotation. Compound II, $C_{12}H_{20}N_2O_3$, was identical with neohydroxyaspergillic acid [7] (elemental analysis, m. p., mixed m. p., optical rotation, and infrared spectra of sublimed samples).



(I) R = Bu^i
(II) R = $CHPr^i \cdot OH$
(III) R = Bu^s
(IV) R = $CMeEt \cdot OH$
(V) R = $CMe_2 \cdot OH$

(VI) R = R′ = Bu^i
(VII) R = CHMeEt, R′ = Bu^i
(VIII) R = R′ = Me

(IX)

(X)

In this paper, derivatives of (VI)—(VIII) are deliberately mis-numbered to agree with that in (I)—(V), in order to facilitate comparison with derivatives of aspergillic acid.

Compounds I and II gave red colours with ferric chloride and green salts with copper sulphate, and had ultraviolet spectra similar to those found for aspergillic acid and related compounds.[5,7] The infrared spectra of these two compounds, when recrystallised from aqueous methanol, as well as those of aspergillic acid and hydroxyaspergillic acid (solid in KBr) included sharp bands at 2030, 2880, 2980 and 3140 cm.$^{-1}$ with broad bands centred at *ca.* 3340 and 2450 cm.$^{-1}$. Since similar bands were found in spectra of muta-aspergillic acid [5] and neohydroxyaspergillic acid,[7] it appeared likely that the region of the infrared

[1] White and Hill, *J. Bacteriol.*, 1943, **45**, 433.
[2] Dutcher, *J. Biol. Chem.*, 1947, **171**, 321; Newbold, Sharp, and Spring, *J.*, 1951, 2679.
[3] Menzel, Wintersteiner, and Rake, *J. Bacteriol.*, 1943, **46**, 109; Dutcher, *J. Biol. Chem.*, 1958, **232**, 785.
[4] Czillag, *Acta Microbiol. Acad. Sci. Hung.*, 1954, **1**, 321.
[5] Nakumura, *Bull. Agric. Chem. Soc.* (*Japan*), 1960, **24**, 629; Nakumura, *Agric. Biol. Chem.* (*Japan*), 1961, **25**, 658.
[6] Nakumura, *Bull. Agric. Chem. Soc.* (*Japan*), 1959, **23**, 65.
[7] Weiss, Strelitz, Flon, and Asheshov, *Arch. Biochem. Biophys.*, 1958, **74**, 150.
[8] Dunn, Newbold, and Spring, *J.*, 1949, 2586.
[9] Kluyver, Van der Walt, and Van Triet, *Proc. Nat. Acad. Sci.*, 1953, **39**, 583; Cook and Slater, *J.*, 1956, 4130, 4133; MacDonald, *Canad. J. Chem.*, 1963, **41**, 165.

spectrum from 4800—2000 cm.$^{-1}$ was characteristic for compounds closely related to aspergillic acid. The band at 2080 cm.$^{-1}$ is interesting in that it lies in the region of C≡C, C≡N, N=C=O stretching, but none of these groups is present in aspergillic acid. However, this band was absent from the spectra of sublimed compound II and of aspergillic acid in chloroform solution. It is probably due to the crystal structure or some type of hydrogen bonding.

Nuclear magnetic resonance (n.m.r.) spectra of compounds I and II, as well as aspergillic acid and related compounds, were obtained (see Table) in order to determine the probable

Nuclear magnetic resonance chemical shifts in the pyrazine series at 60 Mc./sec., with trifluoroacetic acid as solvent and tetramethylsilane as internal reference.

Compound	Chemical shift (τ)	No. of protons *	J (c./sec.)	Assignment †
2-Methylpyrazine	6·88	3·0		2-Me
	0·60	3·0	6	5- & 6-H
	0·50			3-H
2,5-Dimethylpyrazine	7·00	6·0		C–Me
	0·78	2·0		C–H
3,6-Dimethyl-2-hydroxypyrazine (VIII)	7·33	3·0		3-Me
	7·10	3·0		6-Me
	2·37	1·0		5-Me
Deoxyaspergillic acid (VII)	8·94, 8·81	9·0	6	Three (β-Me)
	8·57, 8·45	3·0	6	One (α-Me)
	8·23, 8·12, 8·00	4·0		
	7·87—7·09			
	6·93, 6·82	2·0	8	3-CH_2
	2·34	1·0		5-H
Aspergillic acid (III)	8·98, 8·85	9·0	6	Three (β′-Me)
	8·55, 8·43	3·0	6	One (α-Me)
	8·21—7·48	3·0		
	6·90, 6·79	2·0	8	3-CH_2
	6·62, 6·50, 6·38, 6·27	1·0		6-αH
	2·17	1·0		5-H
Hydroxyaspergillic acid (IV)	8·94, 8·81	9·3	6	Three (β-Me)
	8·06	2·7		One (α-Me)
	7·79, 7·68, 7·55	2·8		
	6·86, 6·74	1·8	2	3-CH_2
	1·70	0·8		5-H
Flavacol (VI)	8·95, 8·83	11·8	6	Four (β-Me)
	7·90, 7·80, 7·68	2·2		
	7·58, 7·47			
	7·27, 7·16	1·9	8	6-CH_2
	6·93, 6·82	1·9	8	3-CH_2
	2·32	0·9		5-H
Compound I	8·93, 8·83	12·0	6	Four (β-Me)
	7·61	2·0		
	7·03, 6·90, 6·80	4·0	8	3- and 6-CH_2
	2·15	1·0		
Compound II	8·92, 8·81	12·2	6	Four (β-Me)
	7·50	1·9		
	6·87, 6·75	1·9	8	3-CH_2
	4·60, 4·52	0·9	5	
	1·78	0·9		5-H
Pulcherriminic acid (IX)	8·97, 8·85	11·9	6	Four (β-Me)
	7·60	2·0		
	6·90, 6·78	4·0	8	3- and 6-CH_2
L-Leucine anhydride (X)	8·95, 8·86	11·8	6	Four (β-Me)
	8·08, 8·00	6·0	5	
	5·46	2·0		NH·C*H*R·CO
	1·20	2·0		Two NH

* Determined from the relative areas of the peaks.
† α and β denote the carbon atoms of the 3-isobutyl side-chain.

structure of the two compounds. Compound II was not appreciably soluble in deuteriochloroform at room temperature but all the compounds investigated were soluble in trifluoroacetic acid, which was therefore used as solvent. The general appearance of the

spectra of aspergillic, hydroxyaspergillic, and deoxyaspergillic acid was the same in deuteriochloroform as in trifluoroacetic acid, but the τ values of the peaks were slightly higher when deuteriochloroform was used. The τ values for pulcherriminic acid differ from those reported by MacDonald [9] because of a shift in spectrum obtained when tetramethylsilane is used as an internal reference rather than as an external reference.

The n.m.r. spectra showed that the aromatic protons of pyrazines absorbed at 0·5—0·8 τ and of hydroxypyrazines (including deoxyaspergillic acid, aspergillic acid, hydroxyaspergillic acid, and flavacol) at 1·7—2·37 τ. The one proton absorption at 2·15 or 1·78 τ for compound I or II, respectively, may therefore indicate one aromatic proton in a hydroxypyrazine ring.

Protons in methyl groups attached directly to the pyrazine or hydroxypyrazine ring absorbed at *ca.* 7 τ. Protons in methyl groups one carbon removed from the ring (in the side chain of aspergillic or hydroxyaspergillic acid) absorbed at *ca.* 8·5 τ, while those in methyl groups two carbons removed (in the isobutyl side chain of flavacol, pulcherriminic acid, and L-leucine anhydride) absorbed at *ca.* 8·9 τ. The twelve-proton doublet with τ values of *ca.* 8·93 and 8·82 for compounds I and II indicates the presence of four β-CH_3 groups in these compounds.

These findings, together with the elemental analysis, indicate that compound I contains two isobutyl side chains, and that it is 2-hydroxy-3,6-di-isobutylpyrazine 1-oxide (I). Reduction to the pyrazine (VI) showed this structure to be correct.

The findings that compound II (neohydroxyaspergillic acid) is related to aspergillic acid in its structure (infrared and ultraviolet spectra) and contains one aromatic proton on the ring and four β-CH_3 groups, and its elemental analysis, indicate that it is similar to compound I, but has a hydroxy-group on one of the side chains. The one-proton doublet centred at 4·56 τ, together with the optical activity of compound II, indicates the presence of the grouping $-CH(OH)\cdot CHMe_2$, and eliminates the alternative possibility, $-CH_2\cdot CMe_2OH$, which should give rise to a two-proton singlet and a compound which would be optically inactive. There are two possible structures for compound II, depending on whether the hydroxylated side chain is at position 3 or 6. Since hydroxyaspergillic and muta-aspergillic acid are both hydroxylated on the side chain at position 6, it seems likely that neohydroxyaspergillic acid has structure (II) and this is supported by the n.m.r. results. The two-proton doublet centred at *ca.* 6·85 τ in the spectra of deoxyaspergillic, aspergillic, and hydroxyaspergillic acid is assigned to the CH_2 protons in the isobutyl side chain at position 3. Further oxidation at N^4 apparently does not change the chemical shift of this CH_2 group, as is shown by the four-proton doublet at 6·84 τ in the spectrum of pulcherriminic acid. In the spectrum of flavacol, the doublet centred at 6·87 τ is therefore assigned to the CH_2 protons in the 3-isobutyl side chain, and the doublet centred at 7·22 τ to the CH_2 protons in the 6-isobutyl side chain. In the spectrum of compound I, as a result of a different environment, the doublet due to the CH_2 group at the 6-side chain is shifted to 6·97 τ and overlaps the doublet at 6·85 τ owing to the CH_2 group attached to C-3, so that what appears to be a triplet is found in the spectrum. In the spectrum of compound II, the doublet centred at 6·81 τ is assigned to the CH_2 protons in the 3-isobutyl group (as in the cases above), so that the hydroxylated side chain is probably at position 6. Therefore compound II is probably 2-hydroxy-6-(1-hydroxy-2-methylpropyl)-3-isobutylpyrazine 1-oxide (II).

Experimental

Melting points were determined on a Fisher–Johns apparatus. Infrared spectra were determined on potassium bromide discs by using a Perkin-Elmer model 21 instrument; n.m.r. spectra were recorded on a Varian A 60 instrument; and ultraviolet spectra were obtained by using Beckman spectrophotometers, models DK2 and DU. Molecular weights were determined by isothermal distillation with acetone as solvent.

Isolation of Compounds I *and* II.—*Aspergillus sclerotiorum* Huber (NRRL 415, PRL 1930)

was maintained on slants of potato–dextrose agar (Difco). A loopfull of spores was used to inoculate 50 ml. of sterile 2% yeast extract medium (Difco) contained in a 250 ml. Erlenmeyer flask. Forty flasks prepared in this manner were incubated at 25° in the dark. The culture liquid in one flask was periodically tested for the production of aspergillic acids, by examining the ultraviolet spectrum in the region of 328 mμ, after dilution with 0·067M-disodium hydrogen phosphate. When a constant value was reached (7—8 days), the contents of the flasks were filtered through glass wool, and the mycelium was washed with small amounts of water. The filtrate and washings were collected together, adjusted to pH 3 with concentrated hydrochloric acid, and extracted with chloroform (6 × 100 ml.), with centrifugation each time to break the emulsion. The chloroform layer was extracted with freshly prepared 0·1M-aqueous sodium hydrogen carbonate (6 × 25 ml.) followed by 0·1M-aqueous sodium carbonate (6 × 25 ml.). The sodium hydrogen carbonate and sodium carbonate layers were separately acidified to pH 3 with concentrated hydrochloric acid and extracted with chloroform. The chloroform layers were dried ($MgSO_4$), and the chloroform was removed, giving from the sodium hydrogen carbonate layer 350 mg. of crude compound II and from the sodium carbonate layer 290 mg. of crude compound I.

Compound II (*Neohydroxyaspergillic Acid*) (II).—The crude sample was a yellow powder, m. p. 155—159°. It was purified by sublimation at 120°/0·02 mm., followed by recrystallisation from aqueous methanol, forming light yellow needles, m. p. 170—171° (yield 60%), $[\alpha]_D^{25}$ −57° (*c* 0·64 in EtOH) $\lambda_{max.}$ (in EtOH) 235 and 328 mμ (ε 8250 and 8940). It was soluble in methanol and ethanol and sparingly soluble in hexane, chloroform, and water. Compound II was dimorphic. The infrared spectrum (solid in KBr) of compound II, recrystallised from hexane, was identical with that of authentic neohydroxyaspergillic acid supplied by Dr. U. Weiss, but was different when recrystallised from aqueous methanol (Found: C, 60·0; H, 8·3; N, 12·0%; *M*, 242. $C_{12}H_{20}N_2O_3$ requires C, 60·0; H, 8·40 N, 11·7%; *M*, 240·3).

The Pyrazine Oxide (I).—The crude material was an almost white powder, m. p. 120—121°. It was purified by recrystallisation from aqueous methanol, giving an almost white powder, m. p. 125—126° (90%), $[\alpha]_D^{25}$ 0° (*c* 0·58 in EtOH), $\lambda_{max.}$ (in EtOH) 236 and 328 mμ (ε 9150 and 10,500) (Found: C, 64·5; H, 9·0; N, 12·6%; *M*, 229. $C_{12}H_{20}N_2O_2$ requires C, 64·3; H, 9·0; N, 12·5%; *M*, 224·3).

Reduction of Compound I.—The compound (150 mg.) was dissolved in glacial acetic acid (5 ml.) and red phosphorus (40 mg.) and iodine (40 mg.) were added. The mixture was boiled under reflux at 150—160° for 8 hr., filtered hot through a sintered funnel into cold 1% sodium bisulphite solution (50 ml.), cooled in ice, and filtered. The solid was sublimed at 120°/0·02 mm. and the sublimate crystallised from aqueous methanol, giving long colourless needles, m. p. 150—151° (80 mg.) $\lambda_{max.}$ (in EtOH) 229 and 325 mμ (ε 8300 and 8450).

Synthesis of 2-*Hydroxy*-3,6-*di-isobutylpyrazine* (*Flavacol*).—L-Leucine (45 g.) was converted into flavacol (2·3 g.) by the procedure of Dunn, Newbold, and Spring.[8] The synthetic flavacol had m. p. 150—151°, $\lambda_{max.}$ (in EtOH) 229 and 325 mμ (ε 8250 and 8320) (lit.,[8] m. p. 144·5—147°). This compound was identical (infrared spectrum and mixed m. p.) with the substance obtained by the reduction of compound I.

2-*Hydroxy*-3,6-*dimethylpyrazine*.—3-Chloro-2,5-dimethylpyrazine (10·7 g.) was heated under reflux with 25% aqueous potassium hydroxide (50 ml.) for 50 hr. The solution was adjusted to pH 6 with concentrated hydrochloric acid and extracted with chloroform. The chloroform layer was dried ($MgSO_4$) and evaporated under reduced pressure. The residue crystallised from aqueous ethanol as fine white needles, m. p. 215° (7·3 g.) $\lambda_{max.}$ (in EtOH) 226 and 320 mμ (ε 6000 and 5630) (lit.,[10] m. p. 208—210°).

The authors thank Dr. U. Weiss for a sample of neohydroxyaspergillic acid, and Wyandotte Chemical Corp., Michigan, U.S.A. for the gift of samples of 2-methylpyrazine, 2,5-dimethylpyrazine and 3-chloro-2,5-dimethylpyrazine. They are indebted to Dr. R. H. Haskins for maintaining the culture, to Mr. M. Mazurek for microanalysis, to Mr. D. M. Tenaschuk for technical assistance, and to Mr. W. C. Haid for infrared spectra.

Prairie Regional Laboratory, National Research Council,
Saskatoon, Sask., Canada. [*Received, March 29th*, 1963.]

[10] Baxter and Spring, *J.*, 1947, 1179.