217. *Production of Antibiotics by Fungi. Part IV. Lateritiin-I, Lateritiin-II, Avenacein, Sambucinin, and Fructigenin.*

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The isolation and degradation of *lateritiin-I, lateritiin-II, avenacein, sambucinin, and fructigenin* are described.

It was observed (Cook, Cox, Farmer, and Lacey, *Nature*, 1947, **160**, 31) that metabolism solutions obtained from *F. lateritium* (two strains), *F. avenaceum*, *F. sambucinum*, and *F. fructogenum* inhibited the growth of *Mycobacterium phlei* at a limiting dilution of 1 : 20 but were only weakly active against *Staphylococcus aureus*. These fungi were studied more closely in view of the possibility of the active principles inhibiting the pathogen, *M. tuberculosis*. The results now communicated were presented at the XIth International Congress of Pure and Applied Chemistry held in London in July, 1947.

The antibiotics were produced by the fungi in the presence of a nitrogen source (" Bactotryptone " or " Eupepton "), glucose, and inorganic salts, and could be extracted with organic solvents such as light petroleum or ether. Preliminary experiments showed the active principles to be insensitive to heat and acid, but sensitive to alkali. Crude material from an ether extract of the medium (pH = 5·5) was purified by chromatography, although, in general, light petroleum extracts afforded material which, after treatment with charcoal, could be purified by crystallisation from aqueous methanol. In this manner the pure antibiotics *lateritiin-I*, m. p. 121—122°, *lateritiin-II*, m. p. 125°, *avenacein*, m. p. 139°, *sambucinin*, m. p. 86—87°, and *fructigenin*, m. p. 129°, were isolated; the m. p. of each antibiotic was depressed on admixture with any one of the others. *Sambucinin* was chromatographed on neutral alumina in order to determine whether it was a mixture of compounds but no further purification was effected.

All five antibiotics contained only carbon, hydrogen, oxygen, and nitrogen, and were neutral compounds. Analyses and molecular-weight determinations on samples from several batches indicated the following formulæ for the antibiotics: lateritiin-I, $C_{26}H_{46}O_7N_2$; lateritiin-II, $C_{26}H_{46}O_7N_2$; avenacein, $C_{25}H_{44}O_7N_2$; sambucinin, $C_{24}H_{42}O_7N_2$; fructigenin, $C_{26}H_{44-46}O_7N_2$. The five compounds contained neither active hydrogen atoms nor methoxyl groups; lateritiin-I, avenacein, and fructigenin contained two *N*-methyl groups and at least four *C*-methyl groups.

They showed ultra-violet absorption only below $\lambda = 219$ mμ. The stability of the compounds to heat and acid was exemplified by lateritiin-I which only lost 10% of its activity on distillation in a tube over an open flame, and 50% of its activity on heating with concentrated nitric acid at 100° for 1 hour. Although dilute caustic alkali caused rapid inactivation at room temperature, greater stability was exhibited towards ammonia, morpholine, and other bases. Complete saturation was demonstrated by means of neutral permanganate, bromine in chloroform, and unsuccessful catalytic hydrogenation.

Vigorous acid hydrolysis of lateritiin-I yielded a solution which on ether extraction afforded a colourless acid, m. p. 65—66°, in the form of plates. Water solubility, a yellow colouration with dilute ferric chloride solution, and sensitivity to oxidising agents suggested the presence of an α-hydroxy-acid, since a β-hydroxy-acid would have formed a halogeno-paraffin under the conditions of hydrolysis (Perkin, *J.*, 1896, **69**, 1484) and a γ- or δ-hydroxy-acid would have lactonised. Microelectrometric titrations, molecular-weight determinations, and analyses of the acid and its benzylamine salt indicated a formula, $C_5H_{10}O_3$. The acid was optically active, $[\alpha]_D$ −19·4° ±2·6° (c = 0·95% in chloroform) and, on oxidation with lead tetra-acetate, a reaction which verified the location of the hydroxyl group on the α-carbon atom, yielded an aldehyde which was volatile in steam and characterised as its 2 : 4-dinitrophenylhydrazone, *p*-nitrophenylhydrazone, and 2 : 4-dinitrophenylsemicarbazone. Analysis, and mixed m. p.s of each of the three derivatives with authentic *iso*butaldehyde derivatives identified the acid as one of the optical isomers of α-hydroxy*iso*valeric acid :

$$CHMe_2{\cdot}CH(OH){\cdot}CO_2H \xrightarrow{Pb(OAc)_4} CHMe_2{\cdot}CHO.$$

Although both forms of α-hydroxy*iso*valeric acid had been previously prepared (Fischer and Scheibler, *Ber.*, 1908, **31**, 2897; Bartlett, Kunz, and Levene, *J. Biol. Chem.*, 1937, **118**, 508) no m. p.s had been recorded; therefore specimens of the two enantiomorphs were obtained by deaminating D- and L-valine with nitrous acid (Fischer and Scheibler, *loc. cit.*). It has been demonstrated that in this reaction no inversion occurs and a hydroxy-acid, with the same configuration as the amino-acid, is obtained (Clough, *J.*, 1918, **113**, 526; Freudenberg and Rhino, *Ber.*, 1942, **57**, 1547). The results summarised in the table below show that D-α-hydroxy*iso*valeric acid is identical with the degradation acid.

	$[\alpha]_D$ ($CHCl_3$).	$[\alpha]_D$ (N-NaOH).	$[\alpha]_D$ (H_2O).	M. p.	Mixed m. p.
D-α-Hydroxy*iso*valeric acid	−19·9° ± 1·8°	+10·8° ± 2·0°	−3·2° ± 1·8°	64·5°	65·0°
L-α-Hydroxy*iso*valeric acid...	+16·9° ± 2·0°	−11·1° ± 2·0°	+2·9° ± 2·0°	65°	81—82°
Acid under investigation.....	−19·4° ± 2·6°	+12·7° ± 1·8°	−2·3° ± 2·0°	65—66°	—

The yields of D-α-hydroxy*iso*valeric acid from the hydrolysis approximated to 2 mols. per mol. of lateritiin-I.

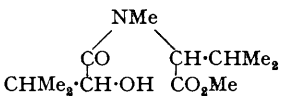
Evaporation of the acid hydrolysate, after ether extraction, gave a colourless gum which showed a tendency to crystallise on vigorous drying. Careful precipitation with ether from an ethanol solution afforded colourless needles, m. p. 149°, $[\alpha]_D^{18°}$ +25·5° ±1·1° (c = 1·75% in ethanol), of a base hydrochloride. Electrometric titrations and analyses indicated a molecular formula $C_6H_{13}O_2N$,HCl, and the presence of a weak acidic grouping. The failure to obtain a positive ninhydrin reaction or effervescence with nitrous acid with the free base, m. p. 265°, were in accordance with further analyses which showed the presence of one *N*-alkyl group. The evidence suggested a methylamino-acid, and samples of the crude and purified base were treated with sodium hypobromite and the products decomposed with steam (Langheld, *Ber.*, 1909, **42**, 2366). In both cases only one carbonyl compound was formed, as demonstrated by the chromatography of its 2 : 4-dinitrophenylhydrazone, and it was identified as *iso*butaldehyde (2 : 4-dinitrophenylhydrazone, m. p. 168°; 2 : 4-dinitrophenylsemicarbazone, m. p. 204°; *p*-nitrophenylhydrazone, m. p. 130°). From the degradation, methylamine was also isolated as its hydrochloride, m. p. 217° (mixed m. p. 219°). The final proof of the homogeneity of the methylamino-acid was obtained by chromatographing a specimen of the crude material on a strip of paper and development with aqueous methanolic cupric acetate; one band only was found. The isolation of *iso*butaldehyde proved that the methylamino-acid was one of the optically active forms of *N*-methylvaline, and on comparison with other methylamino-acids it appeared that the acid probably had an L-configuration. A sample of *N*-methyl-L-valine was prepared from L-valine (Fischer and Lifschitz, *Ber.*, 1915, **48**, 363), and the hydrochloride found not to give a depression of m. p. with the base hydrochloride under discussion. It was also obtained by treatment of L-α-bromo*iso*valeric acid with aqueous methylamine solution and was

again identical with the material of natural origin. Yields of *N*-methyl-L-valine from hydrolyses approximated to 2 mols. per mol. of antibiotic. Lateritiin-I, lateritiin-II, avenacein, fructigenin, and sambucinin formed D-α-hydroxy*iso*valeric acid and *N*-methyl-L-valine on acid hydrolysis in similar yields. In these hydrolyses small amounts of *iso*butaldehyde were detected but these were demonstrated to be formed by D-α-hydroxy*iso*valeric acid alone, under identical conditions.

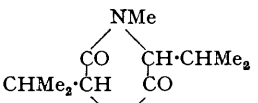
The rapid inactivation of lateritiin-I with dilute sodium hydroxide at room temperature afforded another method of degradation. Electrometric titrations on solutions of lateritiin-I which had been allowed to stand with this reagent for increasing periods of time, indicated the formation of an acidic grouping, the amount of which was proportional to the decrease in activity. The final titration corresponding to total inactivation gave an equivalent of 247, and the simple nature of the curve indicated that two molecules of the acid were formed from one of the antibiotic. Initial experiments resulted in the isolation of a gummy acid which could not be crystallised and failed to give crystalline derivatives. It was observed that this material lost its acidic properties, on standing at room temperature for 3 days, to give a colourless, neutral oil, a change which was accelerated by distillation in a high vacuum. By following the alkali inactivation polarimetrically it was found that, whereas lateritiin-I, the sodium salt of the degradation acid, and the free acid had comparable specific rotations, that of the last gradually changed from $-93°$ to $+167°$ at pH 1·5, demonstrating the occurrence of a chemical change. This change was shown to be a ring closure with loss of water and not a decarboxylation. In two hours the acid was converted quantitatively into the neutral product at 100°. Analysis of the *product*, indicated a formula $C_{11}H_{19}O_3N$, and on acid hydrolysis D-α-hydroxy*iso*valeric acid and *N*-methyl-L-valine were obtained. By reaction of a sample of the free acid with diazomethane a crystalline *methyl* ester, $C_{12}H_{23}O_4N$, was prepared and characterised as the *α-naphthylamine* adduct of the 3 : 5-dinitrobenzoyl derivative. Alkaline hydrolysis of sambucinin and fructigenin gave an acid of which the methyl ester was identical with the corresponding compound from lateritiin-I.

The same ester was obtained by hydrolysis of the lactone with dilute alkali followed by treatment with diazomethane, and must on the above evidence be regarded as the methyl ester of D-α-hydroxy*iso*valeryl-*N*-methyl-L-valine (I), while the lactone is 2 : 5-diketo-4-methyl-3 : 6-di*iso*propylmorpholine (II). These structures were verified by comparison with synthetic materials, obtained by condensing L-α-bromo*iso*valeric acid chloride with *N*-methyl-DL-valine and replacement of the bromine in the product with a hydroxyl group by boiling with pyridine and water. The acid formed lost water spontaneously when kept, and on separation of the two disastereoisomers of the lactone by chromatography on acid alumina, a product was obtained identical in all respects with the natural material. This yielded a crystalline methyl ester, m. p. 75°, on hydrolysis and reaction with diazomethane, which had the same physical properties as the natural ester and gave no depression of melting point on admixture.

The differences in the structures of lateritiin-I, lateritiin-II, avenacein, sambucinin, and fructigenin are attributed to the presence of small fragments in the antibiotic molecules which are yet unidentified. Selective *in situ* oxidation experiments (Williams and Woods, *J. Amer. Chem. Soc.*, 1937, **57**, 1408) on acid hydrolysates failed to show the presence of this fragment, as did attempts to isolate a volatile product from alkaline inactivations. In the latter instance the titration curves obtained in earlier studies exclude the possibility of an acidic or basic fragment.



(I.)



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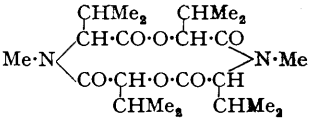

(III.)

Since the announcement of these results in 1947, Plattner and Nager (*Helv. Chim. Acta*, 1948, **31**, 665) have described the degradation of the antibiotic enniatin B, $C_{22}H_{38}O_6N_2$, m. p. 175—175·5°. Acid hydrolysis of this compound afforded two molecules each of D-α-hydroxy*iso*valeric acid and *N*-methyl-(+)-valine; a compound formulated as D-α-hydroxy*iso*valeryl-*N*-methyl-(+)-valine methyl ester was obtained on esterifying the acid obtained by degradation with alkali. No other degradation products were found and the antibiotic was given the structure (III).

The m. p. of enniatin A, $C_{24}H_{42}O_6N_2$, was not depressed on admixture with lateritiin-I and the two compounds might appear to be identical from *X*-ray examination. Enniatin A, however, afforded *N*-methyl-(+)-leucine on degradation, and Plattner and Nager suggest that the valine

derivative obtained from lateritiin-I arises as a gross impurity. In view of the data reported in the present paper this suggestion cannot be accepted, for, in addition, the crude degradation product was submitted to paper chromatography and showed no sign of heterogeneity. Moreover, the structure proposed by the Swiss workers must be viewed with reserve; the antibiotics described in the present paper give rise to degradation products which so far have been unvarying, and as enniatins A and B, lateritiins-I and -II, avenacein, sambucinin, and fructigenin are obviously similarly constituted, acceptance of formula (III) would imply one structure to represent several demonstrably different antibiotics.

Experimental.

The fungi were grown on a medium prepared by dissolving Eupepton No. 2 (100 g.), glucose (100 g.), and sodium chloride (50 g.) in tap water (10 l.), and distributed in quart-size milk bottles (each containing 400 c.c.). Before inoculation the medium was sterilised at 15 lbs. pressure of steam for 15 minutes, and the plugged bottles placed in an almost horizontal position in the incubating room. After inoculation the fungi were allowed to grow for 13—14 days at 25°, by which time the medium had reached maximum antibacterial titre.

Extraction of Lateritiin-I.—The active medium (18 l.) was extracted three times with light petroleum (3 × 1·5 l.), and the extract dried (Na_2SO_4). On evaporation, an almost colourless gum was obtained which was dissolved in methanol (20 c.c.), filtered, and the solution heated to boiling with norite (200 mg.). The filtrate (15 c.c.) was diluted with water (85 c.c.) and allowed to stand for 15 hours. Colourless plates were deposited (754 mg.), m. p. 110—112°. Repeated crystallisation from aqueous methanol gave *lateritiin*-I, m. p. 121—122°, $[\alpha]_D^{20°}$ −95·6° ± 2·0° (*c* = 1% in ethanol) (Found, on different samples: C, 62·77, 62·92, 62·13, 62·41, 62·77, 62·66, 62·90, 62·57; H, 9·23, 9·08, 9·24, 9·00, 8·98, 9·24, 9·02, 9·12; N, 5·92, 6·12, 5·99, 6·05, 6·03, 6·30; *C*-Me, 13·9; *N*-Me, 5·7; *M*, cryoscopic in camphor, 523. $C_{26}H_{46}O_7N_2$ requires C, 62·66; H, 9·24; N, 5·62; 5*C*-Me, 15·1; 2*N*-Me, 6·0%; *M*, 498).

The culture fluids of *F. avenaceum*, *F. sambucinum*, *F. fructigenum*, and *F. lateritium*-II were extracted in a similar manner to those of *F. lateritiin*-I, and the products recrystallised from aqueous methanol. *Avenacein* was obtained in colourless tetrahedra, m. p. 139°, $[\alpha]_D^{19°}$ −101° ±2·0° (*c* = 1% in ethanol) (Found, on different samples: C, 62·08, 61·98, 62·03, 61·77, 61·98, 61·87, 61·84, 62·00; H, 8·93, 8·91, 8·92, 8·80, 8·87, 8·89, 8·81, 8·91; N, 6·03, 5·85, 6·17, 5·83, 5·83; *C*-Me, 14·6; *N*-Me, 5·8; *M*, cryoscopic in camphor, 489, 492, 494. $C_{25}H_{44}O_7N_2$ requires C, 61·98; H, 9·09; N, 5·78; 5*C*-Me, 15·5; 2*N*-Me, 6·2%; *M*, 484).

Lateritiin-II crystallised in colourless plates, m. p. 125°, $[\alpha]_D^{19°}$ −92·0° ±1·6° (*c* = 1·2% in ethanol) (Found, on different samples: C, 62·37, 62·70, 62·40, 62·06, 62·28, 62·47, 62·57; H, 9·07, 9·13, 9·15, 9·08, 8·97, 8·98, 9 22; N, 6·10, 6·09, 6·07. $C_{26}H_{46}O_7N_2$ requires C, 62·66; H, 9·2; N, 5·62%).

Sambucinin was originally obtained as a crude product, m. p. 71—78°. A sample was crystallised five times from aqueous methanol to afford colourless plates, m. p. 86—87°, $[\alpha]_D^{21°}$ −83·2° ±2·0° (*c* = 1% in ethanol) (Found, on different samples: C, 61·18, 61·20, 60·95, 61·18; H, 9·15, 9·29, 9·40, 9·20; N, 5·81. $C_{24}H_{42}O_7N_2$ requires C, 61·20; H, 8·93; N, 5·96%).

Fructigenin crystallised in colourless plates, m. p. 129°, $[\alpha]_D^{18°}$ −103° ±2° (*c* = 1% in ethanol) (Found, on different samples: C, 63·13, 62·92, 62·96, 63·15; H, 8·80, 8·73, 8·89, 8·73; N, 5·78, 5·49, 5·55, 5·63; *C*-Me, 12·6; *N*-Me 5·6. $C_{26}H_{46}O_7N_2$ requires C, 62·66; H, 9·24; N, 5·26; 4*C*-Me, 12·1; 2*N*-Me, 6·0. $C_{26}H_{44}O_7N_2$ requires C, 62·95, H, 8·88; N, 5·65; 4*C*-Me, 12·1; 2*N*-Me, 6 0%).

Extraction of Lateritiin-I from the Mycelium of F. lateritium-I.—The mycelium from 20 l. of medium was well washed with water, dried by suction, and heated at 50° for 48 hours. A brittle mass (135 g.) was obtained which was ground to a powder and extracted with light petroleum to yield a pale amber solution which on evaporation afforded a brown oily gum. The gum was extracted with boiling methanol (20 c.c.), and the solution decolourised with norite (0·4 g.) and diluted with water (80 c.c.). Colourless irregular plates (102 mg.) were formed, m. p. 112°. On recrystallising three times from aqueous methanol lateritiin-I, m. p. 121°, was obtained. Many other samples of dried mycelium yielded no antibiotic and the mycelium was usually not treated as a source of lateritiin-I.

Extraction of Fructigenin from the Mycelium of F. fructigenum.—The mycelium from 30 l. of culture fluid was pressed mechanically to remove most of the water and pulped with hot acetone (4 × 500 c.c.). On evaporation in a vacuum, a pasty mass was obtained which was extracted with chloroform, and the extract was chromatographed on neutral alumina. 1 G. of a biologically active fraction, m. p. 89°, was obtained which on repeated recrystallisation from methanol–water yielded fructigenin, m. p. 128—129° (0·5 g.). On another occasion the yield of antibiotic was much smaller.

Vigorous Acid Hydrolysis of Lateritiin-I.—(A) *Isolation and identification of* D-α-*hydroxy*isovaleric *acid.* Lateritiin-I (675 mg.) was sealed in a glass tube with hydrochloric acid (5 c.c.) and heated to 125° in an oil-bath for 5 hours. After cooling, the contents of the tube were diluted with water (8 c.c.) and extracted three times with ether (3 × 10 c.c.). The ethereal extract was dried and evaporated to give a pale brown gum (370 mg.) which crystallised on scratching. Recrystallisation from benzene–light petroleum gave colourless plates which when pure had m. p. 65—66°, $[\alpha]_D^{13°}$ −21·2° ±1·6° (*c* = 1·25% in chloroform) (Found: C, 51·0; H, 8·6. Calc. for $C_5H_{10}O_3$: C, 50·85; H, 8·5%). The *benzylamine* salt melted at 134° (Found: C, 64·1; H, 8·3; N, 6·4. $C_5H_{10}O_3,C_7H_9N$ requires C, 64·0; H, 8·4; N, 6·2%).

Oxidation of D-α-*hydroxy*isovaleric *acid with lead tetra-acetate* (cf. Oeda, *Bull. Chem. Soc. Japan*, 1934, **9**, 8). D-α-Hydroxy*iso*valeric acid (37 mg.) was dissolved in A.R. glacial acetic acid (3 c.c.) containing lead tetra-acetate (139 mg.), and the mixture kept at 50—60° under reflux for 1¼ hours. The reaction solution was cooled, diluted with ice water (5 c.c.), and adjusted to pH 7·5 with solid sodium hydrogen carbonate. On steam-distillation a distillate was obtained which gave a precipitate with 2 : 4-dinitrophenylhydrazine in 2N-hydrochloric acid. Chromatography of the precipitate yielded deep yellow laths, m. p. 168°, of the dinitrophenylhydrazone (Found: C, 47·5; H, 4·8. Calc. for $C_{10}H_{12}O_4N_4$:

C, 47·6; H, 4·8%). A further oxidation yielded an aqueous distillate from which a *p*-nitrophenylhydrazone, m. p. 130°, and a 2 : 4-dinitrophenylsemicarbazone, m. p. 204°, were prepared. Admixture with the corresponding *iso*butaldehyde derivatives led to no depression of m. p.

Preparation of D-α-*hydroxyisovaleric acid* (Fischer and Scheibler, *Ber.*, 1908, **31**, 2897). D-Valine (1 g.) was dissolved in 2N-sulphuric (16 c.c.) and treated with a solution of sodium nitrite (0·9 g.) to give D-α-hydroxy*iso*valeric acid (545 mg.), m. p. 65—66°, $[\alpha]_D^{14°}$ −19·9° ±1·8° (*c* = 1% in chloroform).

L-α-Hydroxy*iso*valeric acid (470 mg.) was similarly prepared from L-valine (1 g.), and obtained in colourless plates, m. p. 65°, $[\alpha]_D^{14°}$ +16·9° ± 2·0° (*c* = 1% in chloroform).

(B) *Isolation and identification of* N-*methyl*-L-*valine.* The acid solution from the acid hydrolysis of lateritiin-I (675 mg.), after ether extraction, was evaporated to dryness to give a colourless gum (524 mg.) which on intensive drying crystallised slowly. By dissolving the gum in dry ethanol, diluting with sodium-dried ether, and " seeding ", colourless needles were obtained, together with some gum. The latter was recrystallised in the same manner to give a total yield of 372 mg. Fractional crystallisation of the impure *hydrochloride* revealed the presence of only one distinct compound, m. p. 149°, $[\alpha]_D^{18°}$ +25·5° ±1·1° (*c* = 1·75% in ethanol) (Found: C, 43·3; H, 8·1; N, 8·5; *N*-Me, 16·9. $C_6H_{13}O_2N$,HCl requires C, 43·0; H, 8·4; N, 8·4; *N*-Me, 17·3%).

The free N-*methyl*-L-*valine* was obtained by two methods. (1) The powdered hydrochloride (100 mg.) was treated with a slight excess of ethereal diazomethane for 2 minutes and the excess of diazomethane rapidly taken off under reduced pressure; the powdery white solid (78 mg.) which remained was crystallised from aqueous acetone and yielded colourless plates which sublimed at 250—260° and melted at 265° (Found: C, 55·0; H, 9·75. $C_6H_{13}O_2N$ requires C, 54·95; H, 9·9%).

(2) The gummy hydrochloride (110 mg.) was dissolved in water and shaken with silver sulphate (140 mg.) for 1 hour. The solution was filtered, made just alkaline with dilute baryta, and refiltered. The filtrate was adjusted to pH 7 with dilute sulphuric acid, and, after centrifuging, the supernatant liquid was evaporated to dryness. Crystallisation of the residue from aqueous acetone gave colourless plates (62 mg.), m. p. 265°.

Paper-chromatography of the Crude Methylamino-acid.—A sample of the crude methylamino-acid (30 μg.) was chromatographed at 25° on a strip of Whatman No. 1 filter paper, 35 × 2 cm. After 18 hours the phenol had flowed 28 cm. down the paper strip, which was then removed and " dried " in a steam oven. By spraying the chromatogram with 75% aqueous methanolic copper acetate solution one band only was observed.

Oxidation of the Crude N-*Methyl*-L-*valine with Sodium Hypobromite* (cf. Langheld, *Ber.*, 1909, **42**, 2366).—Crude *N*-methyl-L-valine hydrochloride was dissolved in water (5 c.c.), and the solution adjusted to pH 7·5 with dilute sodium hydroxide. To this solution at 0° was added N-sodium hypobromite (2·1 c.c.), and the mixture allowed to stand for 30 minutes, then cooled to −10° and dropped into a current of steam during the course of 3 minutes. The distillate was collected in a cooled flask containing 2N-hydrochloric acid (5 c.c.). Distillation of the acid solution separated the aldehyde from the amine hydrochloride. The aqueous aldehyde was converted into its 2 : 4-dinitrophenylhydrazone (262 mg.) and *p*-nitrophenylhydrazone (90 mg.), which were separately chromatographed on alumina to give one crystalline derivative in each case. The 2 : 4-dinitrophenylhydrazone, m. p. 168°, formed deep yellow laths (Found: C, 47·8; H, 4·2. Calc. for $C_{10}H_{12}O_4N$: C, 47·6; H, 4·7%), and the *p*-nitrophenylhydrazone formed orange needles, m. p. 130°. These compounds when admixed with the corresponding *iso*butaldehyde derivatives showed no depression of m. p.

The amine hydrochloride (60·5 mg.) was obtained on evaporation of the acid solution, and crystallisation from dry ethanol–ether afforded colourless, hygroscopic needles, m. p. 217°. When admixed with methylamine hydrochloride (m. p. 220°) the mixture melted at 219°.

The pure methylamino-acid (51 mg.) was oxidised in a similar manner with 1N-sodium hypobromite (0·45 c.c.), and the 2 : 4-dinitrophenylsemicarbazone (16·1 mg.) of the aldehyde prepared. Crystallisation from ethanol–acetic acid yielded dull amber needles, m. p. 204°, which did not depress the m. p. of an authentic sample of *iso*butaldehyde 2 : 4-dinitrophenylsemicarbazone (Found: C, 45·1; H, 4·5. Calc. for $C_{11}H_{13}O_5N_5$: C, 44·75; H, 4·4%).

Preparation of N-*Methyl*-L-*valine from* L-*Valine* (cf. Fischer and Lifschitz, *Ber.*, 1915, **48**, 360).—L-Valine (1 g.) was dissolved in 2N-sodium hydroxide (4·7 c.c.) and treated with toluene-*p*-sulphonyl chloride (3·22 g.) in ether (8·5 c.c.). The *toluene*-p-*sulphonyl*-L-*valine* (1·339 g.) crystallised from water in colourless needles, m. p. 146—147°, $[\alpha]_D^{21°}$ +19·4° ±2·6° (*c* = 1·45% in ethanol) (Found: C, 53·1; H, 6·4. $C_{12}H_{17}O_4NS$ requires C, 53·1; H, 6·3%).

Tosyl-L-valine (1 g.) was dissolved in 2N-sodium hydroxide (6·35 c.c.) and methylated with methyl iodide (0·93 g.) under pressure to give a pale yellow gum (0·98 g.), $[\alpha]_D^{21°}$ −20·8° ±2·1° (*c* = 1·5% in ethanol). The toluene-*p*-sulphonyl-*N*-methyl-L-valine (324 mg.) was hydrolysed by sealing in a tube with fuming hydrochloric acid (*d* 1·19, 5 c.c.) and heating at 100° for 20 hours. The solution was evaporated to dryness and N-*methyl*-L-*valine hydrochloride* (15·1 mg.) was isolated; it had m. p. 146°, $[\alpha]_D^{18°}$ +18·9° ±2·4° (*c* = 0·85% in ethanol) (Found: C, 43·7; H, 7·9. $C_6H_{13}O_2N$,HCl requires C, 43·0; H, 8·3%). Admixture with the natural methylamino-acid hydrochloride raised the m. p. to 148°.

Preparation of N-*Methyl*-L-*valine from* L-α-*Bromoisovaleric Acid.*—L(−)-α-Bromo*iso*valeric acid (Levene, Mori, and Mikeska, *J. Biol. Chem.*, 1927, **65**, 348) (2 g.) was dissolved in ice-cold 33% methylamine solution (30 c.c.) and kept at 30° for 48 hours. N-Sodium hydroxide solution (25 c.c.) was added, and the solution evaporated to dryness in a vacuum. The gummy residue was dissolved in 2N-hydrochloric acid (15 c.c.), extracted with ether (2 × 25 c.c.), and the aqueous layer neutralised with sodium hydroxide. It was again evaporated to dryness, and the residue extracted repeatedly with ethanol (25 c.c.), whereupon *N*-methyl-L-valine (0·4 g.) was obtained. This was treated with aqueous hydrochloric acid, dried in a vacuum, and recrystallised four times from ethanol–ether. A product was obtained, m. p. 148°, $[\alpha]_D^{21°}$ +19·5° ±2° (*c* = 1·0% in ethanol), which gave no depression of m. p. on admixture with the natural amino-acid hydrochloride.

Vigorous Acid Hydrolysis of Avenacein.—Avenacein (200 mg.) was hydrolysed with hydrochloric acid (2 c.c.) in a sealed tube at 120° for 5 hours. Ether extraction of the hydrolysate yielded D-α-hydroxy-

*iso*valeric acid (79 mg.), m. p. 65—66°, $[\alpha]_D^{20°}$ −20·1° ±2° (*c* = 1% in chloroform); mixed m. p. with an authentic sample, 65°. The benzylamine salt, m. p. 134°, was also prepared. The remaining aqueous solution yielded a hydrochloride (121 mg.) which was identical with *N*-methyl-L-valine hydrochloride, m. p. 149°, $[\alpha]_D^{16°}$ +24·8° ±2·1° (*c* = 0·88% in ethanol). When mixed with an authentic sample the m. p. of the hydrochloride was not depressed.

Vigorous Acid Hydrolysis of Sambucinin.—Hydrolysis of sambucinin (203 mg.) with concentrated hydrochloric acid (2 c.c.) afforded D-α-hydroxy*iso*valeric acid (72 mg.), m. p. 65—66°, $[\alpha]_D^{18°}$ −19·5° ±1·8° (*c* = 1·15% in chloroform), and *N*-methyl-L-valine hydrochloride (124 mg.), m. p. 148—149°, $[\alpha]_D^{18°}$ +25·1° ±2·0° (*c* = 1% in ethanol).

Vigorous Acid Hydrolysis of Lateritiin-II.—Lateritiin-II (150 mg.) was hydrolysed with concentrated hydrochloric acid (1·5 c.c.) to give D-α-hydroxy*iso*valeric acid (54 mg.), m. p. 65°, $[\alpha]_D^{20°}$ −18·4° ±2·2° (*c* = 0·90% in chloroform), and *N*-methyl-L-valine hydrochloride (89 mg.), m. p. 149°, $[\alpha]_D^{21°}$ +25·3° ± 1·9° (*c* = 1·16% in ethanol).

Vigorous Acid Hydrolysis of Fructigenin.—Hydrolysis of fructigenin (300 mg.) with concentrated hydrochloric acid (4 c.c.) for 6 hours at 120—125° yielded D-α-hydroxy*iso*valeric acid (98 mg.), m. p. 65°, $[\alpha]_D^{19°}$ −19·1° ±2° (*c* = 1·0% in chloroform), and *N*-methyl-L-valine hydrochloride (160 mg.), m. p. 148°, $[\alpha]_D^{19°}$ +24·1° ±2° (*c* = 0·95% in ethanol).

D-α-Hydroxy*iso*valeric acid (1·470 mg.) was titrated electrometrically with 0·051N-sodium hydroxide: equiv., 129; pK_a' 3·6. Electrometric titration of *N*-methyl-L-valine hydrobromide (2·869 mg.) with 0·051N-sodium hydroxide showed equiv., 226; pK_a' 10·1.

(C) *Mild Alkaline Hydrolysis of Lateritiin-I.*—Lateritiin-I (435 mg.) was dissolved in methanol (5 c.c.) and kept with 2N-sodium hydroxide (2·5 c.c.) at room temperature for 16 hours. The reaction mixture was diluted with water (10 c.c.), extracted three times with ether (3 × 10 c.c.), and then acidified to pH 1·5 with 2N-sulphuric acid. The solution became turbid on acidification but cleared on ether extraction (4 × 10 c.c.). The extract was dried and evaporated at 30° to give a colourless acidic gum (425 mg.). On standing for 3 days at room temperature a portion of the gum lost its acidity. The remainder (355 mg.) was transferred to a small distillation tube and distilled at 75° in high vacuum to give a colourless, viscous, neutral oil (307 mg.) (Found: C, 62·1; H, 8·9; N, 6·5. $C_{11}H_{19}O_3N$ requires C, 62·0; H, 8·9; N, 6·6%).

Quantitative conversion of acid into lactone. A sample of gummy acid (559 mg.) was introduced into a 25-c.c. flask connected with a weighed calcium chloride tube and two carbon dioxide absorption tubes in series; the flask was slowly heated at 105—110° for 2 hours while purified nitrogen was passed through the apparatus. Water (30·3 mg.) and a negligible quantity of carbon dioxide (3·5 mg.) were collected. The gummy acid was completely converted into the lactone (527 mg.), $[\alpha]_D^{20°}$ +81·8° ±1·6° (*c* = 1·26% in ethanol).

Acid hydrolysis of the lactone. The lactone (92 mg.) was hydrolysed with concentrated hydrochloric acid (2 c.c.) in a sealed tube at 125° for 5 hours. From the hydrolysate, D-α-hydroxy*iso*valeric acid (28 mg.), m. p. 65—66°, $[\alpha]_D^{21°}$ −18·2° ±1·7° (*c* = 1·22% in chloroform), and *N*-methyl-L-valine hydrochloride, m. p. 149°, $[\alpha]_D^{21°}$ +17·4° ±2·0° (*c* = 1% in ethanol), were isolated.

Chromatography of the lactone. The lactone (290 mg.) was dissolved in benzene (5 c.c.) and chromatographed on alumina. The eluate yielded a colourless oil (73 mg.) which was apparently the unchanged lactone. When elution was effected with methanol, a hygroscopic solid (132 mg.) was obtained. This substance was apparently the *sodium* salt of the ring-opened lactone (Found: C, 52·1; H, 7·6. $C_{11}H_{20}O_4NNa$ requires C, 52·2; H, 7·9%).

The acid (121 mg.) from an alkaline hydrolysis of lateritiin-I was carefully prepared, care being taken to avoid lactonisation, and allowed to react with excess of ethereal diazomethane. After 2 minutes the ether and excess of diazomethane were removed under reduced pressure, and the resulting oil set aside for 5 minutes, by which time it had set to a mass of crystals (122 mg.). Repeated crystallisations of the product from light petroleum at −10° yielded long colourless needles of the *methyl* ester, m. p. 78°, $[\alpha]_D^{21°}$ −128° ±4° (*c* = 0·43% in chloroform) (Found: C, 58·6; H, 9·5; N, 5·8; active H, 0·39; *M*, cryoscopic in bromoform, 243. $C_{12}H_{23}O_4N$ requires C, 58·8; H, 9·4; N, 5·7; one active H, 0·41%; *M*, 245). The same ester was obtained in exactly similar manner by mild alkaline hydrolysis of sambucinin.

The ester (163 mg.) was dissolved in dry benzene (1·6 c.c.), and 3 : 5-dinitrobenzoyl chloride (152 mg.) added followed by pyridine (0·1 c.c.). The mixture was heated under reflux on the steam-bath for 15 minutes, diluted with ether (20 c.c.), and the ethereal solution shaken first with 0·05N-sulphuric acid (5 c.c.), and then with 1N-sodium hydrogen carbonate solution (5 c.c.). After washing the ether solution with water (3 × 10 c.c.), drying, and evaporating, a gum was obtained which could not be crystallised even after chromatography on acid-washed alumina. The 3 : 5-dinitrobenzoate was therefore dissolved in ether (3 c.c.) and an excess of α-naphthylamine in ether added, whereupon red crystals of the *α-naphthylamine* adduct were deposited (112 mg.). These were recrystallised three times from methanol and gave red, rectangular plates, m. p. 147° (Found: C, 59·7; H, 6·0. $C_{29}H_{34}O_9N_4$ requires C, 59·8; H, 5·8%).

Hydrolysis of the ester (64 mg.) with concentrated hydrochloric acid (1 c.c.) yielded D-α-hydroxy-*iso*valeric acid (21 mg.), m. p. 64—65°, $[\alpha]_D^{22°}$ −17·9° ±2·3° (*c* = 0·78% in chloroform), and *N*-methyl-L-valine hydrochloride (25 mg.), m. p. 147—148°, $[\alpha]_D^{22°}$ ±24·6° ±2·0° (*c* = 1% in ethanol).

The lactone (195 mg.) was dissolved in methanol (3 c.c.) and kept overnight with 2N-sodium hydroxide (1 c.c.) at room temperature. Extraction of the diluted reaction mixture with ether yielded no product, but acidification and extraction afforded a gummy acid which on reaction with diazomethane gave a crystalline ester (155 mg.), m. p. 78°, mixed m. p. 78° (with methyl ester from alkaline hydrolysis of lateritiin-I).

Mild Alkaline Hydrolysis of Fructigenin.—Fructigenin (300 mg.) was dissolved in methanol (5 c.c.) and 1N-sodium hydroxide (5 c.c.) was added. After the mixture had stood for 16 hours at 20°, water (10 c.c.) was added, and methanol removed in a vacuum. The aqueous solution was extracted with ether (2 × 5 c.c.), acidified with 2N-hydrochloric acid, and re-extracted with ether (2 × 10 c.c.). After being washed with water, the solution was dried and excess of diazomethane in ether was added. After standing for 5 minutes, ether was removed in a vacuum, and the residue crystallised on scratching.

Recrystallisation from light petroleum gave colourless needles, m. p. 77°, not depressed on admixture with the compound from lateritiin-I.

Preparation of the Above Lactone and Methyl Ester from L(−)-α-*Bromo*isovaleric *Acid and* N-*Methyl*-DL-*valine*.—*N*-Methyl-DL-valine (1 g.) was suspended in dry chloroform (25 c.c.), and D(−)-α-bromo*iso*-valeryl chloride (1·5 g.) added, followed by dry pyridine (2 c.c.). The mixture became warm and the solid disappeared; after 10-minutes' shaking, the solvent was removed in a vacuum, leaving a yellow oil. Water (10 c.c.) and pyridine (2 c.c.) were added, and the mixture heated at 100° for 1 hour. It was then cooled, acidified, and extracted with ether (2 × 20 c.c.); the ethereal solution was evaporated in a vacuum and heated at 100° for 1 hour at 15 mm. The gum which resulted was dissolved in ether (20 c.c.) and shaken with 1N-sodium hydroxide (5 c.c.) followed by water (5 c.c.), and the ether removed; a gum (1·5 g.) was obtained, $[\alpha]_D^{20°}$ +52·2° ±2° (*c* = 0·95% in ethanol). This was dissolved in ether and passed through a column (25 cm. × 1 cm.) of acid-washed alumina (pH 4). The total eluate (800 mg.) was kept for several days at 0° and a small amount of crystalline material (m. p. 82°) separated; this was filtered off and found to be optically inactive. The residual oil was the required *lactone*, $[\alpha]_D^{20°}$ +79° ±2° (*c* = 1·0% in ethanol) (Found: C, 61·65; H, 9·0; N, 6·71%. $C_{11}H_{19}O_3N$ requires C, 61·97; H, 8·92; N, 6·57%).

The nature of crystalline material was established by repeating the preparation but using DL-α-bromo*iso*valeryl chloride; after the stage of chromatography on alumina, it was the sole product and is one of the two possible diastereoisomers of the lactone. On using D(+)-α-bromo*iso*valeryl chloride, an oily lactone was obtained, $[\alpha]_D^{20°}$ −78° ±2° (*c* = 0·96% in ethanol); on admixture with an equal amount of the natural lactone, the crystalline material (m. p. 82°) was obtained.

The synthetic lactone ($[\alpha]_D^{20°}$ 79° ±2°) (500 mg.) was dissolved in methanol (5 c.c.) and N-sodium hydroxide (5 c.c.) added. After the mixture had been kept at 20° for 16 hours, water (10 c.c.) was added, and the methanol removed in a vacuum. The solution was extracted with ether (5 c.c.), acidified with hydrochloric acid, re-extracted with ether (2 × 10 c.c.), and dried. Diazomethane in ether was added until the solution remained permanently yellow, and after 10 minutes the solvent was removed in a vacuum. The gummy residue crystallised on scratching and was recrystallised three times from light petroleum (b. p. 40—60°), giving colourless needles, m. p. 75°, not depressed on admixture with the natural *ester*, $[\alpha]_D^{19°}$ −128° ±2° (*c* = 1·0% in chloroform) (Found: C, 58·95; H, 9·35; N, 6·2. $C_{12}H_{23}O_4N$ requires C, 58·78; H, 9·39; N, 5·71%).

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