ISARIIN, A NEW DEPSIPEPTIDE FROM ISARIA CRETACEA[1]

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ABSTRACT

One of the metabolic products of the fungus *Isaria cretacea* has been found to yield four different amino acids and a hydroxyacid on acid hydrolysis. The amino acids were identified as glycine, L-alanine, L-valine, and D-leucine, and a quantitative analysis showed them to be present in the molar ratio of 1:1:2:1, respectively. The hydroxyacid was identified as D-β-hydroxydodecanoic acid. These units appear to be combined in a simple cyclic structure by means of peptide bonds and an ester linkage between the hydroxyl of hydroxydecanoic acid and the carboxyl of the C-terminal amino acid. The sequence valine $\rightarrow$ β-hydroxydodecanoic acid $\rightarrow$ glycine has been established.

In an earlier study (1) it was reported that two strains of *Isaria cretacea* van Beyma had been isolated from the heterokaryotic parent organism. The most noteworthy difference between them was the ability of strain A to produce phototropic synnemata under suitable conditions of growth, whereas strain B formed only a highly branched aerial mycelium. Nutritional differences were also discovered, and the ability to develop synnemata was correlated with a low rate of growth.

During an examination of the metabolic products of the two strains a new depsipeptide, which has been named isariin, was isolated. It was found to be present in both strains A and B as well as the parent heterokaryon.

Isariin was obtained as colorless needles, m.p. 249.5–250.5° C, after repeated recrystallization from aqueous ethanol. Analyses and molecular weight estimations indicated the molecular formula $C_{33}H_{59}O_7N_5$. The infrared absorption spectrum provided evidence for ester and secondary amide groups. Isariin possessed neither acidic nor basic functions, but could be hydrolyzed in mild alkaline conditions to the monobasic isariic acid $C_{33}H_{61}O_8N_5$. That the infrared absorption maximum at 1727 cm^{-1} in this product was that of a carboxyl and not the unchanged ester carbonyl was shown by converting it to the sodium salt, when the maximum was shifted to 1603 cm^{-1}.

Vigorous acid hydrolysis of isariin yielded an ether-soluble product which was separated into neutral and acidic fractions. The acid, m.p. 62.8–63.2° C, gave analyses and a neutralization equivalent indicating the molecular formula $C_{12}H_{24}O_3$. The infrared spectrum showed maxima attributable to an aliphatic hydroxyacid, and upon distillation *in vacuo* a product was obtained which had infrared maxima consistent with those of an unsaturated aliphatic acid. This was converted with the uptake of approximately 1 mole of hydrogen, over palladium catalyst, to lauric acid. From the ease with which the hydroxyacid was dehydrated and from the properties of the unsaturated acid the hydroxyl group was thought to be in the β-position. This was confirmed by synthesis and resolution of DL-β-hydroxydodecanoic acid. Fractional crystallization of the D-amphetamine salt of the racemate afforded a levorotatory acid which was indistinguishable from the hydroxyacid of isariin. The dextrorotatory isomer was also separated and on admixture with an equal part of the natural acid or synthetic levorotatory isomer gave a product which was identical with the synthetic DL-racemate. The dextrorotatory isomer was also

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indistinguishable from a sample of authentic L-β-hydroxydodecanoic acid kindly furnished by Dr. K. Serck-Hanssen, who synthesized the compound by anodic chain extension (2) of methyl L-β-acetoxy-γ-carboxybutanoate. Admixture of the authentic L-isomer with the natural acid or the levorotatory isomer obtained by resolution of the racemate gave a product indistinguishable from the racemate. It is therefore concluded that the compound present in isariin is D-β-hydroxydodecanoic acid.

The neutral fraction from acid hydrolysis of isariin was an oil with an infrared maximum at 1765 cm^{-1}, suggestive of a lactone group. Upon alkaline hydrolysis β-hydroxydodecanoic acid was obtained as the only product, indicating that the material was a lactide formed by interesterification of the hydroxyacid.

Paper chromatographic examination of the aqueous solution from the acid hydrolyzate detected glycine, alanine, valine, and leucine. These were separated on a cellulose column; alanine and valine were found to be of the L- and leucine of the D-configuration. Quantitative analysis of the hydrolyzate showed that glycine, alanine, valine, leucine, and β-hydroxydodecanoic acid were present in the ratio 1:1:2:1:1, respectively.

Hydrazinolysis of isariic acid indicated that one of the two molecules of valine possessed a free carboxyl group and must, therefore, have been esterified with the hydroxyacid in isariin. This result was confirmed by subjecting isariic acid to the Dakin–West reaction (3). Vigorous acid hydrolysis of the product showed all four amino acids to be present in equimolecular amounts. Carboxypeptidase also released valine, but no other amino acid. Absence of enzymatic hydrolysis after the removal of valine suggests that the D-leucine may then have been in the penultimate position. It is unlikely that D-leucine was the newly formed C-terminal unit since its initial presence in the penultimate position should have prevented any action by carboxypeptidase on isariic acid. Less probably the lack of further action on the peptide by carboxypeptidase might have been due to the appearance of alanine in the terminal position, thus providing a relatively poor substrate for continued hydrolytic cleavage (4).

Partial hydrolysis of isariin gave an ether-extractable fraction which, on complete hydrolysis, yielded mainly glycine and β-hydroxydodecanoic acid. It was concluded that these two components were directly linked in the original compound. Five ninhydrin-reacting products, in addition to the constituent amino acids, were separated paper chromatographically from the partial hydrolyzate. In each instance they were found to contain all five amino acids and probably also the hydroxyacid. They are presumed to have been formed by ring opening of isariin at the various peptide bonds. For the depsipeptide a partial structure which is supported by the evidence at present available is shown in Fig. 1.

During the early part of this investigation it was noted that analysis of the components in various preparations of isariin did not yield whole numbers of amino acids. In particular, the content of alanine was frequently higher, and of valine lower, than found in samples which had been repeatedly recrystallized from aqueous ethanol. The presence of traces of an amino acid with an R_f corresponding to that of α-aminobutyric acid was also noted. Attempts to separate the crude material into components by paper chromatography were unsuccessful, but these observations suggested that *I. cretacea* produced a mixture of depsipeptides related to isariin, and that the latter was not the major constituent under all conditions of culture.

Depsipeptides containing a β-hydroxyacid are relatively uncommon. To the authors' knowledge, they have been reported only in esperin (5), an antibiotic containing β-hydroxytridecanoic acid obtained from *Bacillus mesentericus*, and in serratamolide,

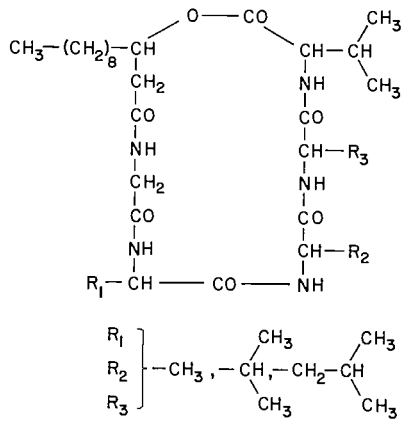

FIG. 1.

recently isolated (6) from *Serratia* strains and shown (7) to possess D-β-hydroxydecanoic acid. β-Hydroxydodecanoic acid appears not to have been previously isolated and unequivocally identified from a natural source, although Bergström and co-workers (8) appear to have obtained some evidence for its presence in the fatty acid components of crude pyolipic acid from *Pseudomonas pyocyanea*.

EXPERIMENTAL

Production of Isariin

In a typical experiment a covered glass tray (9×15 inches) containing 400 ml W.G.A. medium (9) supplemented with neomycin (5 mg) was seeded with the parent strain of *I. cretacea* van Beyma by flooding the surface of the agar with a suspension (27 ml) of a standard spore inoculum (1) and incubated at 28–30° C for 16 days. The mycelium (wet weight 1.1 g) was then scraped from the agar surface, suspended in ethanol (100 ml), and macerated in a Waring blender. The slurry was filtered and the residue re-extracted twice in the same manner. The extracts were combined and evaporated to dryness. The residual solid thus obtained was twice extracted with ethyl acetate (250 ml) under reflux for 30 minutes. The solution was concentrated to dryness and the gummy residue triturated with several portions of petroleum ether, b.p. 30–60° C. The insoluble white solid was crystallized from ethyl acetate as fine needles and constituted crude isariin (18 mg), m.p. 250–252° C. Found: C, 60.84%; H, 8.88%; N, 11.85%.

Strains A and B grown in the same manner yielded 1.6 and 1.0 g of wet mycelium from which 44 and 21 mg, respectively, of crude isariin were isolated.

In an alternative procedure 250-ml Erlenmeyer flasks containing W.G.B. medium (50 ml) were inoculated with 0.5 ml of a standardized spore suspension (1) of strain A incubated at 28–30° C on a rotary shaker for 8 days. The mycelium was then separated and extracted with ethanol as above. The filtrate was extracted with three portions of one-half volume each of *n*-butanol. The ethanolic and butanolic extracts were combined, evaporated to dryness, and the residue worked up, as described above. The yield of crude isariin in a typical experiment was 250 mg/liter of culture medium used.

Purification and Properties

Crude isariin was recrystallized twice from ethyl acetate, and then six times from aqueous ethanol to give fine, colorless needles, m.p. 249.5–250.5° C. Found: C, 62.12%; H, 9.11%; N, 11.14%; molecular weight (Rast in D-borneol) 551, (isothermal distillation in ethanol) 609; saponification equivalent, 638. Calculated for $C_{33}H_{59}O_7N_5$: C, 62.11%; H, 9.32%; N, 10.98%; molecular weight, 637.8. The ultraviolet spectrum in ethanol showed end absorption. Principal infrared maxima (KBr disk) were at 3300, 3060 (w), 2950 (shoulder), 2920, 2855, 1735, 1650 (broad), 1533 (broad), 1470, 1450 (shoulder), 1380 (broad), and 1190 cm^{-1}. Isariin is easily soluble in ethanol, methanol, or chloroform, less readily in acetone, poorly in ether or ethyl acetate, and insoluble in benzene, petroleum ether, water, 2 *N* sodium hydroxide, or hydrochloric acid solution.

Alkaline Hydrolysis of Isariin

Isariin (31.6 mg) was dissolved in methanol (4 ml), and 0.1 *N* KOH solution (1 ml) added. After 48 hours at 40° C the solution was acidified with 0.1 *N* HCl (2 ml) and cooled to 0° C. The gelatinous precipitate was separated, washed with water, and dried. The residue (24 mg) was crystallized twice from aqueous ethanol

as fine needles, melting indefinitely from 194 to 200° C. Found: C, 60.35%; H, 9.37%; N, 10.49%; molecular weight (Rast in D-borneol) 570; neutralization equivalent, 651. Calculated for $C_{33}H_{61}O_8N_5$: C, 60.43%; H, 9.38%; N, 10.68%; molecular weight, 655.9. Infrared maxima (KBr disk) for the free acid were at 2600 (broad, w), 1727, 1635 (broad), and 1543 (broad) cm^{-1}; for the sodium salt at 1655 (broad), 1603, and 1542 (broad) cm^{-1}.

Acid Hydrolysis

Isariin (98.5 mg) was heated for 48 hours at 100° C in a sealed tube with 20% (w/v) HCl (5 ml). The reaction mixture, which contained a brownish oil at the surface, was diluted with water to 20 ml and extracted thoroughly with ether. The ethereal extracts, on evaporation, left a partially crystalline oil (25.8 mg). Calculated for formation of 1 mole of $C_{12}H_{24}O_3$: 33.2 mg. This was separated into acidic (18.7 mg) and neutral (6.8 mg) fractions by distribution between ether and 0.5 *N* Na_2CO_3 solution.

The acidic fraction was crystallized twice from petroleum ether as colorless needles, m.p. 62.8–63.2° C, $[\alpha]_D^{25} - 15.2°$ ($c = 3.4$ in $CHCl_3$). Found: C, 66.42%; H, 11.06%; neutralization equivalent, 218. Calculated for $C_{12}H_{24}O_3$: C, 66.63%; H, 11.18%; molecular weight, 216.3. Principal infrared maxima (KBr disk) were at 3525, 3425, 3090, 2920, 2850, 2660 (broad), 1735, 1706, 1675, 1472, 1436, 1416, 1395, 1365, and 1295 cm^{-1}.

The neutral fraction was hydrolyzed by being heated for 3 hours under reflux in a mixture of ethanol (4 ml) and 4 *N* NaOH (1 ml). The acidic product (7 mg) was crystallized from petroleum ether to give fine needles, m.p. 62.5–63.0° C, undepressed on admixture with the acid obtained directly from hydrolysis of isariin.

The aqueous solution from the acid hydrolysis was evaporated to dryness over solid NaOH. It gave a strongly positive ninhydrin test and paper chromatography in several common solvent systems showed four ninhydrin-reacting substances to be present. These were identified by direct comparison with standards as glycine, alanine, valine, and leucine.

The residue dissolved in a mixture of *sec*-butanol (12 ml) and 3% aqueous ammonia (4 ml) was chromatographed on a column (2.5×60 cm) of powdered cellulose developed with the same solvent system. Fractions (10 ml) were collected and tested for the presence of amino acids by paper chromatography. Appropriate fractions were combined and evaporated to dryness. The residue from each was crystallized from aqueous ethanol and then sublimed under high vacuum for analysis.

The first yielded glistening, colorless plates from aqueous ethanol, $[\alpha]_D^{27} -13.0°$ ($c = 0.67$ in 6 *N* HCl). Found: C, 54.96%; H, 9.84%; N, 10.58%. Calculated for $C_6H_{13}O_2N$: C, 54.94%; H, 9.99%; N, 10.68%. The infrared spectrum (KBr disk) was indistinguishable from that of authentic D-leucine.

The second compound crystallized as colorless plates, $[\alpha]_D^{28} +30.0°$ ($c = 0.61$ in 6 *N* HCl). Found: C, 51.34%; H, 9.50%; N, 11.96%. Calculated for $C_5H_{11}O_2N$: C, 51.26%; H, 9.47%; N, 11.96%. The infrared spectrum (KBr disk) was indistinguishable from that of authentic L-valine.

The third compound crystallized as colorless prisms, $[\alpha]_D^{27} +17.0°$ ($c = 0.56$ in *N* HCl). Found: C, 40.44%; H, 7.60%; N, 15.49%. Calculated for $C_3H_7O_2N$: C, 40.44%; H, 7.92%; N, 15.72%. The infrared spectrum (KBr disk) was indistinguishable from that of authentic L-alanine.

The fourth compound crystallized as colorless needles, m.p. 232° C (decomp.). Found: C, 32.10%; H, 6.54%; N, 18.43%. Calculated for $C_2H_5O_2N$: C, 32.00%; H, 6.71%; N, 18.66%. A mixed melting point with authentic glycine showed no depression, and the infrared spectra (KBr disk) of the two substances were indistinguishable.

Lauric Acid

When the ether-soluble acid from the acid hydrolysis of isariin was distilled *in vacuo* (10^{-2} mm Hg) in a sublimation block at a block temperature of 100° C a discrete fraction was collected which, on cooling in an ice bath, formed a white crystalline solid, m.p. 25–26° C, with infrared maxima (film) at 2920, 2850, 2680 (broad), 1695, 1650, 1470, 1423, 1380, and 983 cm^{-1}. The substance took up hydrogen equivalent to 0.91 mole/mole, calculated on the basis that it was dodecenoic acid, and yielded an acid, m.p. 40–41° C, which gave a single peak on gas–liquid partition chromatography, using a column containing diethylene glycol succinate as liquid phase, with the same retention time as lauric acid. A mixture with authentic lauric acid, m.p. 42.5–43° C, gave m.p. 41.5–43° C. The infrared spectra of the two substances were indistinguishable.

Synthesis and Resolution of β-Hydroxydodecanoic Acid

Ethyl β-ketododecanoate was prepared in a yield of 70% by condensing caproyl chloride with the sodio derivative of ethyl acetoacetate and hydrolyzing the intermediate with sodium methylate in methanol, according to the procedure of Hunsdiecker (10). The keto ester was hydrogenated for 2½ hours at 1600 p.s.i. and 300° F over Raney nickel, as described by Skogh (11), hydrolyzed with alcoholic KOH under reflux, and the product crystallized from petroleum ether (b.p. 60–80° C) to yield DL-α-hydroxydodecanoic acid, m.p. 68.3–69.2° C, in an overall yield of 47% based on caproic acid.

To the racemic acid (1.22 g) in ether (120 ml) was added a solution of D-amphetamine prepared by treating D-amphetamine sulphate (2 g) with *N* NaOH (20 ml) and extracting the base into ether (25 ml). The precipitated salt was redissolved by adding just sufficient ethanol to the refluxing mixture, and allowed

to cool slowly to 10° C. The mass of fine needles (776 mg, m.p. 125–129° C) which separated was removed by filtration and recrystallized twice from ether–ethanol to give 360 mg of material of m.p. 119–121° C. The free acid was regenerated by shaking the product with dilute HCl and ether; the ethereal solution was dried over anhydrous sodium sulphate and evaporated to dryness. The residue, after crystallization from petroleum ether (b.p. 60–80° C), gave needles (172 mg), m.p. 62.5–63.25° C, $[\alpha]_D^{25}$ $-15.2(\pm 1)°$ (c = 1.6 in $CHCl_3$). Found: C, 66.38%; H, 10.94%. A mixture of equal parts of this substance and the hydroxyacid from isariin melted at 62.5–63.25° C. The infrared spectra (KBr disks) of the two compounds were indistinguishable.

The mother liquor from the initial crystallization of the D-amphetamine salt was cooled to −40° C to yield a crop of feathery needles (581 mg) melting mainly at 78–79° C. After two recrystallizations from ether at low temperature the material (495 mg) gave a sharp m.p. of 78–79° C. The free acid, regenerated as before, yielded 249 mg of small needles, m.p. 61–63.25° C, $[\alpha]_D^{25}$ $+14.4(\pm 1)°$ (c = 2.8 in $CHCl_3$). Found: C, 66.70%; H, 11.28%. The infrared spectrum of a sample in a KBr disk, prepared by evaporating an ethereal solution to dryness on KBr powder, was identical with that of the levorotatory isomer examined similarly.

No depression of melting point was observed when a sample was mixed with authentic L-β-hydroxydodecanoic acid, m.p. 62.2–63.2° C, $[\alpha]_D^{25}$ $+15.6(\pm 1)°$ (c = 2.1 in $CHCl_3$), and the two samples had virtually identical infrared spectra (KBr disk). When admixed with an equal amount of the hydroxyacid from isariin and recrystallized from petroleum ether (b.p. 60–80° C), the compound melted at 69–69.75° C. A similar mixture of authentic L-β-hydroxydodecanoic acid with the natural acid melted at 68–69.5° C. Mixtures of this compound or the authentic L-isomer with the levorotatory product from resolution of the racemate melted at 68.7–69.7° C and 69–69.75° C, respectively. All such mixtures of compounds with opposite optical rotations gave no depression when admixed with the unresolved racemate, and had infrared spectra (KBr disks from ether) which were indistinguishable from each other and from the racemate, but different from that of the optically active substances.

A comparison of authentic L-β-hydroxydodecanoic acid with the acids from isariin and from resolution of the racemate carried out by Dr. Serck-Hanssen yielded results similar to those obtained in this laboratory.

Quantitative Amino Acid Analysis

A sample of isariin (6.00 mg), hydrolyzed in a sealed tube with 25% w/v HCl at 110° for 2½ days, was extracted with ether, the aqueous phase freed of excess hydrochloric acid and made to 2 ml with water. Measured amounts of this solution were applied in triplicate to paper strips and chromatographed in *n*-butanol – acetic acid – water (4:1:1) at the same time as mixtures containing known amounts of leucine, valine, alanine, and glycine. The developed chromatograms were dried, treated with ninhydrin, the density of the spots measured with a densitometer, and the amount of each amino acid estimated by comparison with the standards, according to the procedure of Redfield and Guzman Barron (12).

The values obtained for a sample of crude isariin showed the content of glycine, alanine, valine, and leucine to be (to the nearest 0.05 mole/mole) 0.95, 1.30, 1.75, and 0.90, respectively. In addition, there was a trace of an amino acid with R_f values in several solvent systems corresponding to those of α-aminobutyric acid. For preparations of isariin purified by repeated crystallization from aqueous ethanol the content of amino acids was 1.0, 0.95, 2.05, and 1.0 mole/mole, respectively.

Hydrazinolysis of Isariic Acid

Isariic acid (1.5 mg), hydrazine sulphate (30 mg), and hydrazine (1 ml) were heated in a sealed tube at 60° C for 16 hours. Excess hydrazine and hydrazides were removed by the method of Bradbury (13). Paper chromatography of an aliquot of the aqueous solution showed only a single ninhydrin-reacting substance with R_f values in several solvent systems corresponding to valine. When the remainder of the solution was treated with 1-fluoro-2,4-dinitrobenzene and the dinitrophenylamino acids were separated and paper chromatographed in *n*-butanol – 3% aqueous ammonia (9:1), a spot corresponding in R_f value with 2,4-dinitrophenylvaline was obtained.

Dakin–West Reaction

The procedure described by Turner and Schmerzler (14) was used. Isariic acid (2.5 mg) was heated in a sealed tube at 135° C with pyridine (0.25 ml) and acetic anhydride (0.625 ml). The contents of the tube were then evaporated to dryness and hydrolyzed by heating under reflux with 6 *N* HCl for 24 hours. Excess acid was removed in a desiccator containing solid NaOH, and the amino acid composition of the product estimated quantitatively by paper chromatography as described above. Glycine, alanine, valine, and leucine were present in the ratio 1.0:1.0:1.05:1.0.

Action of Carboxypeptidase on Isariic Acid

Isariic acid (4.75 mg) was dissolved in water (4.5 ml) by adding 0.1 *N* NH_4OH to pH 9.0. A suspension of carboxypeptidase (2 mg) in water (0.1 ml) was added, the pH adjusted with dilute acetic acid to 7.8, and the final volume made up accurately to 5 ml with water. The suspension was agitated gently at room temperature and aliquots removed for quantitative paper chromatographic amino acid analysis at 0.5, 1, 2, 4, 8, and 24 hours. The only amino acid detected was valine in amounts of 0.58, 0.62, 0.73, 0.75, 0.86, and 0.88 mole/mole of isariic acid.

Partial Acid Hydrolysis of Isariin

Isariin (10 mg) was dissolved in glacial acetic acid (1.5 ml), and concentrated HCl (1.5 ml) added. The solution was kept at 40° C in a stoppered flask. At intervals, 0.5-ml aliquots were removed, evaporated to dryness, and the residue distributed between equal volumes of ether and water. At 4 days the ether-soluble fraction contained no products reacting with ninhydrin, but gave a strong positive test after further hydrolysis of the material with 6 *N* HCl under reflux for 18 hours. Paper chromatographic examination showed this to be due mainly to glycine, although small amounts of alanine, valine, and leucine were also present. After 6 days, only glycine could be detected in the hydrolyzate of this fraction. Paper chromatography of the aqueous fraction showed small amounts of all four amino acids present after 4 days with traces of ninhydrin-reacting material having R_f values higher than leucine in all solvent systems examined. The proportion of the latter substances appeared highest after 6 days. No compounds with R_f values lower than leucine, other than single amino acids, were detected during the 10-day period in which the hydrolyzate was examined.

The remaining water-soluble products from all sampling times were combined and separated by paper chromatography on a preparative scale. The mixture was applied as a line along the origin of a sheet of Whatman 3 MM paper and the chromatogram developed ascendingly with *n*-butanol – pyridine – 3% w/v aqueous ammonia (2:1:1). Marker strips cut from each edge of the paper and treated with ninhydrin showed, in addition to the expected single amino acids of R_f values 0.09 (gly), 0.16 (ala), 0.27 (val), and 0.37 (leu), five distinct purple zones at R_f values of 0.50, 0.58, 0.65, 0.78, and 0.87. Segments corresponding to these were cut from the untreated paper and eluted with 50% aqueous methanol. Each was evaporated to dryness and the residue hydrolyzed with 20% w/v HCl in sealed tubes for 24 hours at 110° C. The hydrolyzates all gave the characteristic odor associated with the presence of β-hydroxydodecanoic acid and, upon paper chromatographic examination, were found to contain all of the four amino acids present in isariin.

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