Microbial Conversion of 2,2-Diphenyl-3-(1-pyrrolidino)propionitrile*

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ABSTRACT: *Penicillium* species A-9 NRRL 3946 formed three major microbial transformation products from 2,2-diphenyl-3-(1-pyrrolidino)propionitrile. Two of the products were isolated in crystalline form and identified. The third proved to be unstable and not isolable. One product was identified as 2,2-diphenyl-3-(1-pyrrolidino)propionamide. The second product was identified as hexahydro-3,3-diphenylpyrrolo[1,2-*a*]-pyrimidin-2-[1*H*]one and represents a novel microbial synthesis of this ring system.

2,2-Diphenyl-3-(1-pyrrolidino)propionitrile (I) (Figure 1) was synthesized by a Mannich-type reaction of diphenylacetonitrile with formaldehyde and pyrrolidine. This compound and others were prepared and tested as analogs of the analgesic Methadon (Zaugg *et al.*, 1953).

2,2-Diphenyl-3-(1-pyrrolidino)propionitrile was selected as a typical candidate for microbial conversion studies because its structure suggested ready susceptibility to microbial attack. Approximately 300 identified mold, basidiomycete, and actinomycete cultures were screened for their ability to transform this substrate. The fermentation medium and culture conditions were similar to those previously reported (Theriault and Longfield, 1967). Surprisingly, 2,2-diphenyl-3-(1-pyrrolidino)propionitrile was highly refractive to microbial attack. However, one culture, *Penicillium* sp. A-9 NRRL 3946 formed three major microbial conversion products. This paper describes the isolation and identification of two of these products.

Materials and Methods

Culture. Penicillium species A-9 NRRL 3946 used in these studies was maintained on nutrient agar slants consisting of glycerol, molasses, peptone, and inorganic salts. Inoculated slants were incubated 7 days at 28°, and then stored in the cold at 4°.

Fermentation Medium. Studies were carried out in 500-ml erlenmeyer flasks containing 100 ml of medium consisting of the following ingredients (grams per liter): glucose monohydrate, 50.0; soybean flour, 5.0; Difco yeast extract, 5.0; KH_2PO_4, 4.1; K_2HPO_4, 0.8; NaCl, 1.0; and tap water to 1000 ml. The pH was adjusted to 6.0. Flasks were plugged with cotton and autoclaved at 120–121° at 15–16 psi for 35 min.

Fermentation Procedures. All flasks were inoculated with one-half of an agar slant culture of *Penicillium* species A-9 suspended in sterile nutrient broth. Inoculated flasks were incubated on a Gump rotary shaker (260 rpm) at 28° for 48 hr. 2,2-Diphenyl-3-(1-pyrrolidino)propionitrile was then added (unless otherwise indicated) in powdered form at a level of 0.1% (100 mg/100 ml of culture). The flasks were then incubated on the shaker and sampled at various ages during the fermentation.

Thin-Layer Chromatography (*tlc*). Aliquots (10 ml) of fermentation beers (liquors) were extracted with ethyl acetate at pH 9. The ethyl acetate extracts were evaporated to dryness under vacuum and reconstituted in 2 ml of methanol. The methanol solutions were applied (200 μl) on 20 × 20 cm glass plates coated with Merck-Darmstadt silica gel GF^{254} 500 μ in thickness. Thin-layer plates were developed in a solvent system consisting of methylene chloride–95% aqueous methanol–water (85:15:1, v/v). After development, thin-layer plates were sprayed with 2 N H_2SO_4 followed by the iodoplatinate spray (Jackson and Moss, 1969). The substrate and related conversion products were revealed as purple spots on a pink background.

Spectral Analyses. Infrared spectra were determined with a Perkin-Elmer Model 421 spectrophotometer. Nuclear magnetic resonance (nmr) spectra were determined with a Varian A-60 and Varian HA-100 spectrometer. Nmr spectra were determined in deuterated chloroform ($CDCl_3$) with Me_4Si as the internal standard. Mass spectra were determined with an Associated Electrical Industries Model M.S. 902 mass spectrometer. Melting points were determined with a Kofler micro hot stage melting-point apparatus. All melting points are corrected.

Product Isolations. Large-scale shaken flask runs (10 l.) were harvested at the predetermined optimal age for peak product yields. The pooled harvest fermentation beer was adjusted to pH 9 with NH_4OH and then extracted with two volumes of ethyl acetate. The ethyl acetate extract was evaporated to dryness under vacuum. The residue obtained was dissolved in methylene chloride, filtered, and chromatographed over a pH 4 acid alumina column. The products were eluted with increasing concentrations of methanol in methylene chloride. Further purification was obtained by preparative tlc. Column fractions were pooled, concentrated, and streaked on 20 × 20 cm glass plates coated with Merck Darmstadt silica gel GF^{254} about 500 μ in thickness. Tlc plates were developed in the described solvent system. Since the ultraviolet (uv) absorption of the substrate and conversion products was low, they were revealed by spraying the edges of the tlc plates with the iodoplatinate spray. Individual components were eluted in methanol, filtered, and further purified. Neutral alumina, Florisil, and silica gel columns were also investigated in subsequent isolations. In each case preparative tlc was required for secondary separations.

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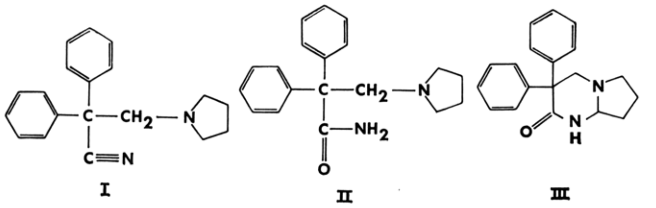

FIGURE 1: Structures of 2,2-diphenyl-3-(1-pyrrolidino)propionitrile (I), 2,2-diphenyl-3-(1-pyrrolidino)propionamide (II), and hexahydro-3,3-diphenylpyrrolo[1,2-*a*]pyrimidin-2[1*H*]-one (III).

2,2-Diphenyl-3-(1-pyrrolidino)propionamide Synthesis. This compound was prepared by reacting 10 g of 2,2-diphenyl-3-(1-pyrrolidino)propionitrile with 50 ml of concentrated sulfuric acid with stirring at 90° for 3 hr. The mixture was then poured into ice and cooled while the pH was adjusted to 7 with 20% aqueous alkaline methanol. The resultant precipitate was filtered, washed with water, and recrystallized from ethanol. The product yield was 6.3 g, mp 139–140°.

Results

As shown in positions 1 and 2 of Figure 2 *Penicillium* sp. A-9 formed three polar (slower moving) microbial conversion products from 2,2-diphenyl-3-(1-pyrrolidino)propionitrile, which were revealed on silica gel GF^{254} thin-layer plates by the iodoplatinate spray as purple spots on a pink background. The most polar product (R_F 0.3) proved to be highly unstable and not isolable. The other two conversion products, II (R_F 0.475–0.50) and III (R_F 0.75–0.775), were barely discernible by 168 hr in the fermentation but increased in intensity with age. Cultures were harvested anywhere from 240 hr up to 528 hr. A number of variables were investigated in order to increase the yields of II and III. Neither of these two conversion products were, of course, produced in the absence of the culture. When the substrate (I) was added to a 48-hr fully grown shaken flask culture of *Penicillium* sp. A-9, further inactivated by placing in a boiling water bath for 10 min or by autoclaving 15 min at 121° and 15–16 psi, none of the microbial conversion products were detected by tlc with up to 528-hr incubation on the shaker. Addition of the substrate (I) to the growing culture at lower levels in powdered form or from a 10% dimethylformamide solution did not improve the yield of either product appreciably. Cells (48-hr old) of *Penicillium* sp. A-9, when washed three times and resuspended in 0.01 M pH 7 phosphate buffer, formed a small amount of apparent III from the substrate (I) added at a level of 0.1% in powdered form. A cell free extract was obtained by sonication of washed cells for 15 min at 20 kc/sec in an ice bath and then finally centrifugation at 12,000 rpm at −5° for 30 min. This preparation failed to form any II or III from 2,2-diphenyl-3-(1-pyrrolidino)propionitrile (I). 2,2-Diphenyl-3-(1-pyrrolidino)propionamide (II) was also added to a growing 48-hr culture of *Penicillium* sp. A-9 but no detectable levels of III were observed. Following isolation and purification by solvent extraction and preparative tlc, the two products (II and III) were each dissolved in methylene chloride, concentrated, and finally precipitated as chromatographically pure white amorphous solids. The II conversion product yield at this point was approximately 5%. Compound III yields were in the range of 1%. Compound II gave needle-like crystals from acetone, mp 145–146°. *Anal.* Calcd for $C_{19}H_{22}N_2O$: C, 77.52; H, 7.53; N, 9.51; O, 5.44. Found: C, 77.67; H, 7.18; N, 9.36; O, 5.58. The uv spectrum in methanol showed λ_{max} at 253 and 259 nm with a shoulder at 265 nm and was quite similar to the starting material (I). The molecular extinction coefficient (ϵ) λ_{max} 259 nm was 482.78. The ir spectrum in chloroform showed the following major absorption frequencies (cm^{-1}): 3450 (m) NH_2, 3200 (m) NH_2, 2975 (s) CH_2, 1675 (s) amide I, 1570 (m) amide II, 1492 (m) C_6H_5, 1133 (m) NH_2. The nitrile band observed at 2220 cm^{-1} in the starting material was absent. A strong carbonyl band was seen at 1675 cm^{-1}. One NH band was present at 3450 cm^{-1}. The amide II band at 1570 cm^{-1} was more like a primary than a secondary amide. The 60-MHz nmr spectrum was essentially the same as that of 2,2-diphenyl-3-(1-pyrrolidino)propionitrile except for the presence of two unusually widely separated NH resonances at 415 and 590 Hz. A tentative structure of 2,2-diphenyl-3-(1-pyrrolidino)propionamide was thus proposed for II. An authentic sample was prepared from 2,2-diphenyl-3-(1-pyrrolidino)propionitrile as described. Both the ir and nmr spectra of the two samples were virtually superimposable, thus establishing the identity of II.

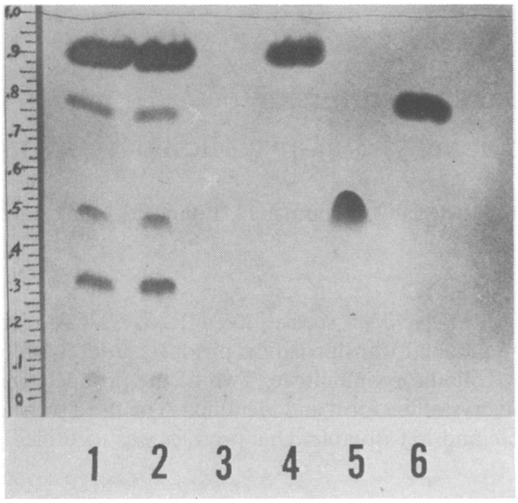

FIGURE 2: Thin-layer chromatography silica gel GF^{254} plate developed in CH_2Cl_2–95% aqueous methanol–H_2O (85:15:1, v/v) for 45 min and sprayed with 2 N H_2SO_4 followed by the iodoplatinate spray. (1) 408-hr extracted fermentation sample; (2) 528-hr extracted fermentation sample; (3) 528-hr extracted control culture (minus substrate) sample; (4) 2,2-diphenyl-3-(1-pyrrolidino)propionitrile (I); (5) 2,2-diphenyl-3-(1-pyrrolidino)propionamide (II); and (6) hexahydro-3,3-diphenylpyrrolo[1,2-*a*]pyrimidin-2[1*H*]-one (III).

The conversion product III (R_F 0.75 in Figure 2) was recrystallized from hot methylene chloride and dried, mp 159–160°. *Anal.* Calcd for $C_{19}H_{20}N_2O$: C, 78.05; H, 6.90; N, 9.58; O, 5.47. Found: C, 78.11; H, 6.88; N, 9.74; O, 5.52. The molecular extinction coefficient (ϵ) λ_{max} 259 nm was 608.1. The ir spectrum in chloroform showed the following absorption frequencies (cm^{-1}): 3449 (w) NH, 3220 (w), 3000 (m) CH_2, 2850 (w), 1672 (s) amide I, 1610 (w), 1498 (m) C_6H_5, 1449 (m), 1418 (m), 1378 (m), 1342 (m), 1297 (m), 1235 (w), 1183 (w), 1142 (m), 1108 (w), 1097 (w), 1033 (w), 1002 (w), 978 (w). One NH band was seen at 3449 cm^{-1}. The amide I band at 1672 cm^{-1} was consistent with a δ lactam, and the absence of an amide II band together with the presence of the NH band further suggested a lactam structure. The phenyl rings were indicated at 1498 and 1610 cm^{-1}.

The 100-MHz nmr spectrum (given in δ) (Figure 3) of this product was measured in $CDCl_3$ with Me_4Si as the internal standard. The complex multiplet centered at 1.76 integrated

TABLE I: Major Spectral Peaks from the Mass Spectrum of Hexahydro - 3,3 - diphenylpyrrolo[1,2 - *a*]pyrimidin - 2[1*H*]one (III).

m/*e*	Rel Abundance (%)	Structure Represented
293	10	M + 1
292	48	M $C_{19}H_{20}N_2O$
194	100	$(C_6H_5)_2C{=}C{=}O$
180	39	$(C_6H_5)_2C{=}CH_2$
166	48	$(C_6H_5)_2C$
165	54	$C_6H_5CC_6H_4$
151.2	Metastable peak	$(C_6H_5)_2C{=}CH_2 \rightarrow C_6H_5CC_6H_4$
142.1	Metastable peak	$(C_6H_5)_2C{=}C{=}O \rightarrow (C_6H_5)_2C$
129	Metastable peak	$C_{19}H_{20}N_2O \rightarrow (C_6H_5)_2C{=}C{=}O$

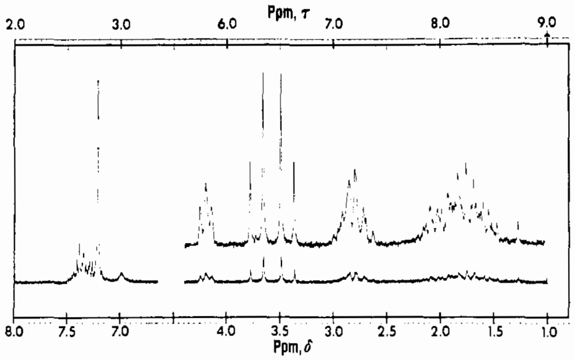

FIGURE 3: 100-MHz nmr spectrum of hexahydro-3,3-diphenylpyrrolo[1,2-*a*]pyrimidin-2[1*H*]-one (III) in $CDCl_3$ with Me_4Si as the internal standard.

for four protons and could be assigned to the 7- and 8-methylene groups. The multiplet at 2.8 was due to the two protons at C-6 of the pyrrolidine ring of the molecule. The incorporation of the C-4 methylene group into a ring system prevented free rotation and the protons became an AB system. The coupling constant (J_{AB} = 12–13 Hz) was acceptable for geminal splitting in rigid systems specified as 12–15 Hz (Jackman, 1959). The chemical shift of the AB quartet (3.57) was consistent with the singlet observed in the corresponding acyclic methylene group of the parent compound. The single proton at C-9 did not couple with the vicinal NH proton but rather with the protons of the 8-methylene group and was thus seen as a triplet at 4.2. This proton was, in addition, deshielded by the two adjacent nitrogen atoms and would be expected to be further downfield than the protons of the 4-methylene group deshielded by only one nitrogen atom. The 1-NH proton was assigned to the broad resonance at 6.98 which exchanged with D_2O. The resonances of the two phenyl ring protons were seen in the range 7.2–7.45 and integrated for 10 protons. The nmr and ir spectra thus supported a tentative structure for compound III of hexahydro-3,3-diphenylpyrrolo[1,2*a*]pyrimidin-2[1*H*]one as shown in Figure 1. The major peaks of the mass spectrum of III are shown in Table I. A molecular ion at 292.1582 was observed (calcd for $C_{19}H_{20}N_2O$: 292.1575). Three metastable transitions were observed. The 151.2 peak was formed by the degradation of the *m*/*e* 180–165. The 142.1 peak was formed by the degradation of *m*/*e* 194–166. The 129 peak was formed in the degradation of the *m*/*e* 292–194 peak. These data confirmed the previously proposed structure.

Discussion

Lang (1894) found that cyanide was excreted from animals as thiocyanate. Lang (1933) studied this enzymatic conversion and isolated the crude enzyme called rhodanese as dried liver powders. This enzyme catalyzed the rapid formation of thiocyanate from cyanide in the presence of sodium thiosulfate or colloidal sulfur at about 38° and pH 8.3. Organic bound cyano or nitrile groups such as acetonitrile, propionitrile, and cyanoacetic acid were, however, unaffected. Bray *et al.* (1951) fed isomers of tolunitrile to rabbits. *m*-Tolunitrile fed at 1-g doses showed one metabolite, *m*-cyanobenzoic acid accounting for most of the compound fed. *p*-Tolunitrile fed at the same level in rabbits showed a 60% conversion to *p*-cyanobenzoic acid, a 10% conversion to *p*-cyanohippuric acid, and a minor hydrolysis of the nitrile group to terephthalic acid (0.8%) and terephthalamic acid (0.9%). *o*-Tolunitrile yielded an 3% conversion to phthalic acid, and a 21% conversion to phthalide. Thimann and Mahadevan (1964) described a nitrilase enzyme which hydrolyzed nitriles to the corresponding carboxylic acids. This enzyme was shown to be present in certain fungi, grasses, cabbages, radishes, and members of the banana group.

Penicillium sp. A-9 showed no chromatographic evidence of hydrolyzing the nitrile group of compound I to the corresponding carboxylic acid. The most polar unstable microbial conversion product (Figure 2 position 1 & 2, R_F 0.3) did not give a typical carboxylic acid response when tlc plates were sprayed with acid–base indicator dyes. 2,2-Diphenyl-3-(1-pyrrolidino)propionamide (II) when added as the sole substrate to growing cultures of *Penicillium* sp. A-9 was not converted to III in levels detectable by thin-layer chromatography. Compound II, thus, does not appear to be an immediate precursor for this novel ring closure in the formation of III.

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