1 ml/min. 10 ml fractions were collected and fractions 35–47 containing neutral amino acids were combined and evaporated to a thick yellow syrup. The syrup was diluted with a small vol. of 1.5 N HCl and run through a column (2 × 100 cm) of Dowex 50 × 8, cation exchange resin (H^+), 200–400 mesh. Amino acids were eluted with 1.5 N HCl at a flow rate of 1 ml/min with 10 ml fractions collected. Fractions 54–59 contained 2,4-*cis*-4,5-*cis*-4,5-dihydroxypipecolic acid and a second uncharacterized imino compound which gave a blue ninhydrin color. These fractions were combined, evaporated to a small vol. and desalted on a small CG-120, (H^+) column. The imino acids were eluted from the column with 2 N NH_3 and concd. The two compounds were separated by ascending PC on Whatman 3MM paper using solvent system 3. The *cis–cis* dihydroxy isomer migrated as the upper band. This was cut from the paper, extracted with a large vol. of H_2O and placed on a small CG-120, (H^+) column to remove residual PhOH. The pure *cis–cis* isomer was eluted from the column with 2 N NH_3, evaporated to dryness and recrystalized twice in aq. EtOH. 270 mg of compound were obtained.

Spectral analysis. Spectral data for both the free and chlorohydrate forms of the *cis–cis* isomer were obtained. IR and NMR spectra of synthesized samples of all four 4,5-dihydroxypipecolic streoisomers were provided by G. Dardenne (Faculté des Sciences Agronomiques, Gembloux, Belgium). The IR spectra for the *cis–cis* isomer showed the appropriate functional group absorptions and the fingerprint region also matched exactly that of the synthetic *cis–cis* compound [6]. NMR spectra (recorded at 100 MHz in D_2O with TSS as a standard) also corresponded closely to those of the synthetic compound. The chlorhydrate spectrum gave characteristic multiplets in the regions 1.9–2.4, 3.1–3.6 and 3.9–4.2 [6].

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N-ACETYL-6-HYDROXYTRYPTOPHAN A NATURAL SUBSTRATE OF A MONOPHENOL OXIDASE FROM *ASPERGILLUS NIDULANS*

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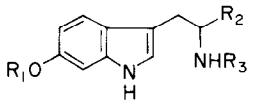
Key Word Index—*Aspergillus nidulans*; mutant; conidiophore pigment; monophenol oxidase; enzyme substrate; *N*-acetyl-6-hydroxytryptophan.

Abstract—An *ivo B* mutant of *Aspergillus nidulans*, deficient in conidiophore pigment has been shown to accumulate *N*-acetyl-6-hydroxytryptophan. This acts as a substrate for a specific monophenol oxidase present in the wild type but absent in the mutant.

INTRODUCTION

Many species of *Aspergillus* have a grey-brown pigment in the upper parts of the conidiophore, including the metulae and phialides [1]. Studies with conidiation mutants of *A. nidulans* [2] have used aconidial mutants, such as *brl A42*, in which these parts of the mycelium are exposed at the colony surface, to select 'ivory' mutants lacking this pigment. These mutants fall into two groups, genetically distinguished as belonging to *ivo A* and *ivo B* loci. Mutants of the latter type are deficient in a specific phenol oxidase [3] and accumulate, at the time of conidiophore formation, a pigment precursor which is the subject of this paper. The *ivo A* mutants possess the phenol oxidase and are assumed to be unable to synthesize the precursor.



1 $R_1 = R_2 = R_3 = H$
2 $R_1 = H$, $R_2 = CO_2H$, $R_3 = COMe$
3 $R_1 = Me$, $R_2 = CO_2Me$, $R_3 = COMe$
4 $R_1 = PhCH_2$, $R_2 = CO_2H$, $R_3 = H$
5 $R_1 = R_3 = H$, $R_2 = CO_2H$
6 $R_1 = H$, $R_2 = CO_2Me$, $R_3 = COMe$

RESULTS AND DISCUSSION

The *ivo B* mutant [4, 5] used lacked conidiophore pigment and was completely deficient in the monophenol oxidase. Preliminary studies showed that this strain, unlike the wild type or simple *brl A*42, mutants, accumulated a compound, soluble in water or methanol but insoluble in nonpolar solvents, which after PC or Si gel TLC could be detected using ferric chloride–potassium ferricyanide [6]. This compound also stained blue with Ehrlich's reagent and bright pink with diazotized sulphanilic acid which suggests the presence of a 6-hydroxyindole moiety [7, 8]. This was supported by the UV spectrum of the purified metabolite, which was very similar to that of 6-hydroxyindole acetic acid [9], and by the NMR spectrum. In this, the aryl proton region was particularly informative, showing the expected pattern of chemical shifts and *ortho*, *meta* and *para* coupling constants and being closely similar to this region in the spectrum of 6-hydroxytryptamine (**1**). The metabolite was deduced to be *N*-acetyl-6-hydroxytryptophan (**2**) from the remaining spectroscopic data and was characterized as its methyl ether methyl ester (**3**). These structures were confirmed by synthesis of **2** and **3** in racemic form from 6-benzyloxy-DL-tryptophan (**4**).

Extracts of *ivo B* strains also showed the presence of traces of another phenolic compound which stained similarly with the same reagents, but which was lost during the purification process. The R_f of this compound on paper using butanol–acetic acid–water was 0.25 (cf. **2**, R_f 0.78) which is close to that of 5-hydroxytryptophan. This suggests that this minor constituent may be 6-hydroxytryptophan (**5**).

Since, in the wild type, the monophenol oxidase is only produced at the time of conidiophore formation, enzyme preparations were conveniently obtained as crude extracts of an *ivo A brl A*42 strain which remain at this stage of development. Both the natural and synthetic samples of **2** were rapidly oxidized by these preparations giving, where sufficient material was oxidized, brown products, suggesting comparison with the pigment formed *in vivo*. A number of related compounds were also tested as possible substrates. Of these, neither 6-hydroxytryptamine (**1**) nor 5-hydroxytryptophan showed any evidence of oxidation and the methyl ester (**6**) (prepared from **4**), was at best a poor substrate, the rate of oxidation being over 150 times slower than that of the natural substrate (**2**).

EXPERIMENTAL

Production and isolation of N-*acetyl-6-hydroxytryptophan* (**2**). Strain G841 of *Aspergillus nidulans* (full genotype *paba A*1; *brl A*42 *ua Y*9 *ivo B*(63), was grown in Petri dishes on agar containing 'complete medium' [5] plus trace elements [10]. After 4 days at 37° the mycelial mat from 50 such cultures was dried and then ground several times with cold MeOH. Evaporation of the combined extracts under red. pres. gave an oil which was triturated with EtOAc to remove fats and other neutral impurities. A soln of the residue in H_2O (30 ml) was satd with $(NH_4)_2SO_4$ and acidified to pH 2–3 with H_3PO_4. Extraction with EtOAc was followed by purification by TLC on Kieselgel H using BuOH–HOAc–H_2O (63:10:27) as eluent. The phenol (**2**) was conveniently located on chromatograms by staining small areas with diazotized sulphanilic acid and was obtained as a colourless glass (2–5 mg); IR ν_{max}^{KBr} cm^{-1}: 3390 *br*, 1710, 1620 *br*, 1550 sh; UV λ_{max}^{EtOH} nm (log ε): 235 (22 000), 267 inf (3700), 276 (3900), 295 (4200); UV $\lambda_{max}^{EtOH+NaOH}$ nm (log ε): *ca* 235 (28 100), 268 inf (5000), 278 inf (4300), 313 (4700); NMR (CD_3OD) δ: 1.90 (3H, *s*, NAc), 3.0–3.42 (2H, *m*, $-\underline{CH_2}CH$), 4.63 (1H, *m*, $-CH_2\underline{CH}N-$), 6.28 (1H, *m*, NH), 6.59 (1H, *dd*, *J* = 2.2, 8.5 Hz, H-5), 6.74 (1H, *dd*, *J* = 0.6, 2.2 Hz, H-7), 6.91 (1H, *br s*, H-2), 6.97 (1H, *m*, indole NH), 7.36 (1H, *dd*, *J* = 0.6, 8.5 Hz, H-4).

Methylation of the metabolite **2** *to give the ester* **3**. Treatment of the above phenolic acid (7 mg) in MeOH with an excess of ethereal CH_2N_2 gave an oil which after prep. TLC on Si gel using EtOAc as eluent gave the methyl ether methyl ester (**3**), R_f 0.3 (orange spot on development with Ce^{4+}, changing to green on heating); UV λ_{max}^{EtOH} nm (log ε): 225 (19 700), 275 (3200), 294 (3500); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3480 (NH), 3440 (NH), 1740 (ester C=O), 1680 and 1550 (–CONH–), 1630, 1500, 1200; NMR ($CDCl_3$) δ: 1.96 (3H, *s*, –COMe), 3.28 (2H, *d*, *J* = 5.3 Hz, $-\underline{CH_2}-CH$), 3.7 and 3.84 (each 3H, *s*, OMe), 4.94 (1H, *dt*, *J* = 5.3, 8 Hz, $D_2O \rightarrow t$, *J* = 5.3 Hz, $-CH_2\underline{CH}NH-$), 6.02 (1H, *br d*, *J* = 8 Hz, exchangeable with D_2O, NH), 6.79 (1H, *dd*, *J* = 1.9, 8.3 Hz, H-5), 6.83 (1H, *s*, H-2), 6.84 (1H, *d*, *J* = 1.9 Hz, H-7), 7.38 (1H, *d*, *J* = 8.3 Hz, H-4), 8.05 (1H, *br s*, exchangeable with D_2O, NH); MS *m/z* 290 $[M]^+$, 231 $[M-CO_2Me]^+$, 216 $[M-CO_2Me-Me]^+$, 200, 189 $[M-CO_2Me-CH_2=C=O]^+$, 176, 174, 160 [base peak, β-(6-methoxyindolyl)CH_2^+], 145, with metastable ions corresponding to the transitions 290 → 231, 231 → 216, 189 → 174 and 160 → 145. (Found: $[M]^+$ at *m/z* 290.12664. $C_{15}H_{15}N_2O_4$ requires 290.12663.)

6-*Hydroxy*-DL-*tryptamine* (**1**). A sample of this was obtained from Sigma as its creatinine sulphate complex. NMR downfield region (CD_3OD, D_2O) δ: 6.71 (1H, *dd*, *J* = 2, 8.5 Hz, H-5), 6.90 (1H, *d*, *J* = 2 Hz, H-7), 7.11 (1H, *s*, H-2), 7.36 (1H, *d*, *J* = 8.5 Hz, H-4).

N-*Acetyl-6-benzyloxy*-DL-*tryptophan* (**4**). 6-Benzyloxy-DL-tryptophan (100 mg) in 1 M NaOH (2 ml) was treated with Ac_2O (3 × 1.7 ml) with vigorous shaking after each addition. The mixture was then warmed to *ca* 40° for 2 hr and allowed to stand at room temp. overnight. The resulting ppt was separated and washed successively with cold H_2O (2 × 3 ml), 1 N HCl (3 ml) and cold H_2O (2 × 3 ml). The crude acetyl derivative (**4**) (81 mg) crystallized from MeOH as a microcrystalline solid, mp 206–208° (lit. [11] mp 208–210°); IR ν_{max}^{nujol} cm^{-1}: 3405 s, 3335, 1725, 1715, 1650 sh, 1636 s, 1559, 1258; NMR ($CDCl_3$) δ: 1.9 (3H, *s*, NAc), 3.20 (2H, *m*, $-\underline{CH_2}CHN-$), 4.70 (1H, *m*, $CH_2\underline{CH}N-$), 5.07 (2H, *s*, $-CH_2O-$), 6.77 (1H, *dd*, *J* = 2.2, 8.6 Hz, H-5), 6.92 (1H, *d*, *J* = 2.2 Hz, H-7), 6.96 (1H, *s*, H-2), 7.28–7.45 (5H, *m*, Ph), 7.43 (1H, *d*, *J* = 8.6 Hz, H-4); MS *m/z* (rel. int.): [352.1426 $[M]^+$ (15.6) $C_{20}H_{20}N_2O_4$ requires 352.1423], 293.1049 $[M\text{-}MeCONH_2]^+$, (6.3), 262.0929 $[M-C_7H_6]^+$ (7.6), 261.0869 $[M-C_7H_7]^+$ (16.1), 236.1064 [β-(6-benzyloxyindolyl)CH_2^+] (75.2), 202.0503 $[M-C_7H_7-MeCONH_2]^+$ (12.9), 146.0599 [β-(6-hydroxyindolyl)CH_2^+] (22.5), 117.0579 [β-(6-hydroxyindolyl)$CH_2-CHO]^+$ (7.4).

N-*Acetyl-6-hydroxyl*-DL-*tryptophan* (**2**). A suspension of *N*-acetyl-6-benzyloxy-DL-tryptophan (**4**, 10.4 mg) and 10% Pd–C

(6.2 mg) in a mixture of EtOH (1 ml) and H_2O (1 ml) was hydrogenated at room temp. until uptake of H_2 had ceased (2 hr). After removal of the catalyst under N_2 using glass paper, evaporation at < 1 mm pressure gave the phenolic indole (**2**) as a colourless glass; IR ν_{max}^{KBr} cm^{-1}: 3410 *s*, 1722, 1630 *s*, 1555; MS *m/z* (rel. int.): [262.0953 $[M]^+$ (14), $C_{13}H_{14}N_2O_4$ requires 262.0953], 203.0581 $[M-MeCONH_2]^+$ (19), 146.0602 [*β*-(6-hydroxyindolyl)CH_2]$^+$ (100), 133.0529 $[M-CH_2=C(NHAc)CO_2H]^+$ (4.8), 117.0574 [*β*-(6-hydroxyindolyl)$CH_2-CHO]^+$ (2.6). This was identical (TLC behaviour, UV, NMR spectra) with a sample of **2** isolated as above.

N-*Acetyl*-6-*methoxy*-DL-*tryptophan methyl ester* (**3**). Methylation of the above phenolic acid (**2**, 5 mg) with ethereal CH_2N_2 gave the ether ester (DL-**3**), R_f 0.3 on TLC using Si and EtOAc (orange spot on development with Ce^{4+} changing to green on heating); IR $\nu_{max}^{CDCl_3}$ UV and NMR spectra identical to those of a sample of the ether ester prepared from the metabolite **2**. (Found: $[M]^+$ at *m/z* 290.1276. $C_{15}H_{15}N_2O_4$ requires 290.1266.)

Preparation of the monophenoloxidase. Cultures of strain AJC 7.46 (*ivo A1 orn B7 brl A42*) or, for the monophenol oxidase free control, strain G 841, were grown as in the production of *N*-acetyl-6-hydroxytryptophan (**2**) but for only 2 days. The resulting mycelial mat was ground in a mortar with sand and Tris–maleic buffer, pH 7.0 at 4°. The crude extract was centrifuged for 5 min at 2500 *g* and in some cases dialysed against the extraction buffer in the cold for 1 hr. Enzyme activity was assayed by adding enzyme preparation (0.1 ml) to a mixture of 0.5 M Tris–maleic buffer, pH 7.0 (1.9 ml), and an aq. soln (1 ml) containing 4-methoxyphenol (25 mg) and catechol (0.2 mg). The formation of the brown oxidation product was followed as *A* at 470 nm in a Unicam SP 1800 spectrophotometer.

Enzymic oxidation of **2**. The oxidation of purified natural **2**, on addition of monophenol oxidase preparations, could be followed polarographically and its destruction could be monitored by chromatography using diazotized sulphanilic acid for detection. Control enzyme preparations from *ivo B* strain, which lack monophenoloxidase were inactive and **2** could still be detected on chromatograms of the mixture.

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TOTAL SYNTHESIS OF SYLVAMIDE, A *PIPER* ALKAMIDE

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Key Word Index—*Piper sylvaticum*; Piperaceae; alkamide; sylvamide; *N*-isobutyl-4,5-dihydroxy-2(*E*)-decenamide.

Abstract—The structure, *N*-isobutyl-4, 5-dihydroxy-2 (*E*)-decenamide, for sylvamide is confirmed by its total synthesis. The *erythro* stereochemistry is also established by comparison of the properties of the natural and synthetic samples.

INTRODUCTION

During the chemical investigation on the seeds of *Piper sylvaticum* Roxb. we isolated a new alkamide, sylvamide (**1**), whose structure was established by spectral studies and degradation experiments [1]. In the present communication we report the confirmation of its structure and the establishment of the relative stereochemistry of the two chiral centres from its total synthesis by a stereospecific pathway.

RESULTS AND DISCUSSION

The scheme formulated for the synthesis required an *N*-