The Identification of 3-Amino-9*H*-pyrido[3,4-*b*]indole Derivatives in L-Tryptophan Pyrolysates

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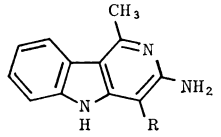
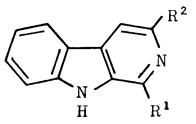
The compounds, 3-amino-9*H*-pyrido[3,4-*b*]indole and 3-amino-1-methyl-9*H*-pyrido[3,4-*b*]indole, which are effectors in induction of sister chromatid exchanges in human cells, were isolated from pyrolysis products of L-tryptophan and characterized by HPLC and UV techniques.

A number of heterocyclic amines in pyrolysates of amino acids, proteins, and proteinaceous foods were found to be highly mutagenic to *Salmonella typhimurium*.[1] Sugimura *et al.*[2,3] reported that the main mutagenic components of pyrolysates of L-tryptophan were 3-amino-1,4-dimethyl- (**1**) and 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (**2**). Compounds **1** and **2** have been synthesized from 2-indolecarboxylic acid and 2,5-lutidine, respectively.[4] The isolation of 2-amino- (**3**) and 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (**4**) as mutagens from the pyrolysis product of tryptophan was also reported.[5] 9*H*-Pyrido[3,4-*b*]indole (**5**, norharman) and its 1-methyl derivative (**6**, harman) were also found in the pyrolysates.[3] Compounds **1** and **2** are carcinogenic to rodents[6] and active in inducing sister chromatid exchanges (SCEs) in human cells.[7]

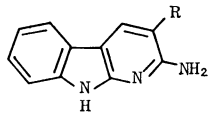
In a previous paper,[8] we reported that 3-amino-9*H*-pyrido[3,4-*b*]indole (**7**) and 3-amino-1-methyl compound (**8**) were relatively weak SCE inducers, and inhibited SCE induction by other stronger SCE inducers. Thus, it is quite interesting to examine whether those compounds **7** and **8** are present in pyrolysates of amino acids and proteins or not. This paper describes the identification of **7** and **8** in L-tryptophan pyrolysates.



1, R=CH_3
2, R=H



5, $R^1=R^2=H$
6, $R^1=CH_3$, $R^2=H$
7, $R^1=H$, $R^2=NH_2$
8, $R^1=CH_3$, $R^2=NH_2$
9, $R^1=NH_2$, $R^2=H$



3, R=H
4, R=CH_3

Results and Discussion

Pyrolysis of L-Tryptophan and Analyses of Products. A one hundred gram sample of L-tryptophan was pyrolyzed and dry-distilled by a direct flame at 400 to 500 °C for 1 h to give a tar (61 g). The tar was separated into acidic, basic, and neutral fractions by the ordinary method to afford 22 g of basic mixture. The mixture was fractionated by a silica-gel column using dichloromethane and a mixture of dichloromethane and ethanol as elution solvents. The fraction expected to contain the title derivatives was further fractionated on a column of silica gel into four subfractions by eluting with a mixture of dichloromethane and ethanol. The material at each step of purification was checked by thin-layer chromatography. Each of the resulting subfractions was subjected to preparative thin-layer chromatography on a silica-gel plate with an appropriate solvent system. The bands corresponding to **2**, **3**, **5**—**8**, and 1-amino-9*H*-pyrido[3,4-*b*]indole (**9**) were made visible under UV light, scraped off, and extracted with methanol. This isolation protocol is summarized in Fig. 1. Each of the methanol extracts was then applied to high performance liquid chromatography (HPLC) to analyze the target compounds, **2**, **3**, **5**—**8**, and **9**, qualitatively and quantitatively.

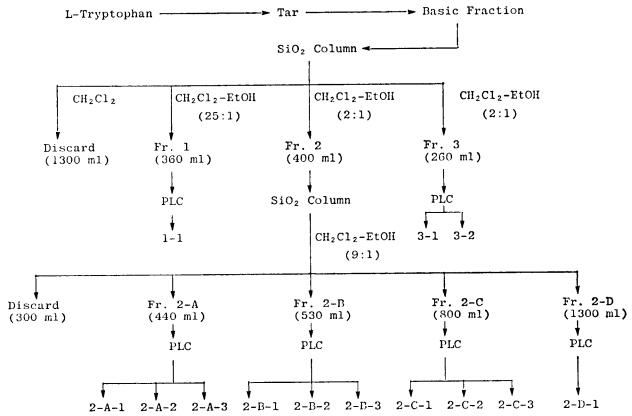


Fig. 1. Protocol for isolation of the target compounds from the pyrolysate.

Typical examples of analytical HPLC of fractions containing the target compounds are shown in Fig. 2. Satisfactory coincidence of retention times of the target bases with those of the authentic specimens was observed. The retention times of the title compounds, **7** and **8**, under various chromatographic conditions were in agreement with the bases of the pyrolysates (Table 1).

Analytical HPLC of Fraction 2-C-2 under the chromatographic condition No. 2 (Table 1) is shown in Fig. 3. Retention times of Peaks I and II coincided with those of **7** and **8**, respectively. Peaks I and II fractions were pooled separately from several identical runs of HPLC. As shown in Fig. 4, the absorption curves in UV of these two peak substances were completely identical to those of synthetic **7** and **8**, respectively. These facts confirm that peaks I and II are

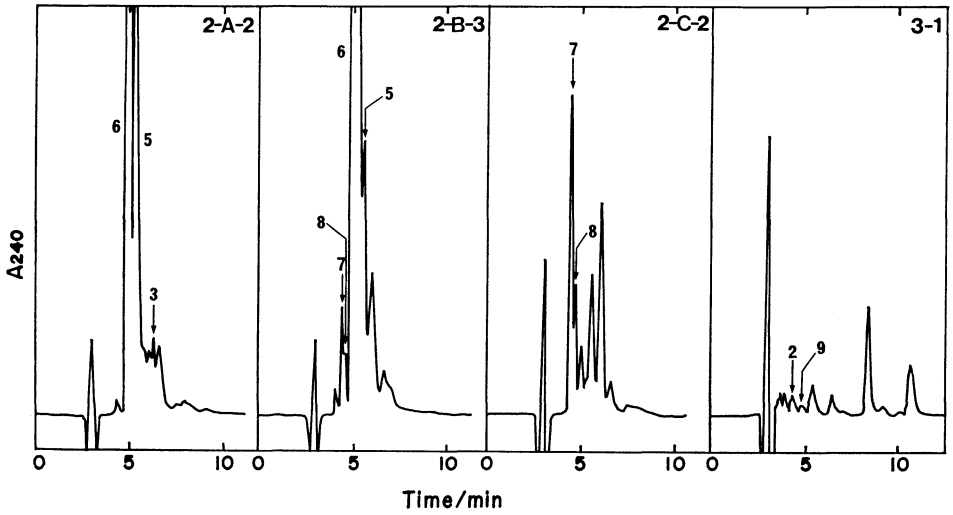


Fig. 2. Analytical HPLC of typical fractions containing the target compounds. The chromatographic conditions are shown as No. 1 in Table 1.

Table 1. Retention times of the target compounds in various HPLC systems

No.	Column	Solvent system	Time/min						
			2	**3**	**5**	**6**	**7**	**8**	**9**
1	μBondapak C_{18}[a]	CH_3CN/0.02 M[b] KH_2PO_4 (45/55)	4.1	6.3	5.0	4.9	4.2	4.4	4.5
2	LS-410 ODS SIL[c]	MeOH/H_2O/NH_4OH (60/40/1)	12.0	9.4	14.2	18.6	6.1	7.2	16.9
3	LS-470 OH SIL[c]	MeOH/H_2O (8/2)					9.3	13.6	
4	LS-410 ODS SIL	CH_3CN/0.02 M KH_2PO_4 (45/55)					6.1	5.9	
5	LS-470 OH SIL	CH_3CN/0.02 M KH_2PO_4 (45/55)					4.0	3.8	

Column size: 4×300 mm. Flow rate: 1.0 ml/min. Detector: UV 240 nm.
a) Waters associate. b) 1 M=1 mol dm^{-3}. c) Toyo Soda Manuf. Co., Ltd.

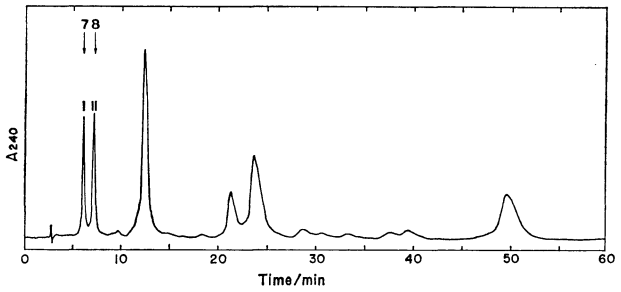


Fig. 3. HPLC of Fraction 2-C-2. Arrows indicate the retention times of **7** and **8**. The chromatographic conditions are shown as No. 2 in Table 1.

of **7** and **8**, respectively. Both **7** and **8** were also detected in fr. 2-B-2. Analyses of other bases of the pyrolysates were worked up in a similar manner to that given above. Compounds **3**, **5**, and **6** were mainly detected in both fr. 2-A-1 and fr. 2-A-2. Compounds **2** and **9** were found in fr. 2-D-1, fr. 3-1, and fr. 3-2. The contents of the target compounds were estimated from HPLC data, ignoring their decomposition and loss during separation, and are shown in Table 2.

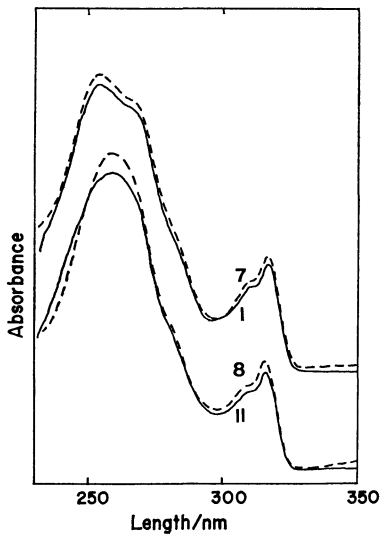


Fig. 4. UV spectra of Peaks I and II (——) and compounds **7** and **8** (----).

Compounds **7** and **8**, in methanol solution were stable at room temperature in the dark, but were considerably photosensitive. At a distance of 20 cm

TABLE 2. PYROLYSATES OF L-TRYPTOPHAN (100 g)

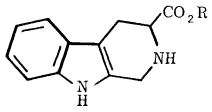
Compound	Content/mg
6	1140—1040
5	852—846
3	249—101
7	12.7—11.9
8	4.7—3.1
9	4.9—0.11
2	2.6—0.28

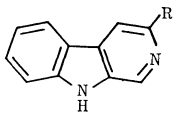
from a 20 W-fluorescent tube the half-life times of **7** and **8** were *ca.* 25 h and *ca.* 12.5 h in methanol, respectively (Fig. 5). From these facts it seems that the original contents of **7** and **8** in the fresh pyrolysates did not differ so much from each other as those seen in Table 2. Compound **2** was stable under these conditions.

The present work shows that the combined use of column and preparative thin-layer chromatography, HPLC, and UV is highly effective for analysis of pyrolysis products.

The identification of the title derivatives in pyrolysates of natural substances are currently under investigation.

*Synthesis of 9H-Pyrido[3,4-*b*]indole Derivatives.* 1,2,3,4-Tetrahydro-9*H*-pyrido[3,4-*b*]indole-3-carboxylic acid (**10**)[9] was treated with methanol and ethanol to afford methyl and ethyl esters (**11** and **12**), respectively. The methyl ester (**11**) was dehydrogened with sulfur to give methyl 9*H*-pyrido[3,4-*b*]indole-3-carboxylate (**13**). Treatment of the ethyl ester (**12**) under similar conditions resulted in dehydrogenation to afford ethyl carboxylate (**14**). When lead(IV) acetate was used, compounds **11** and **12** were also converted into **13** and **14**, respectively, but the yields were poor. Treatment of **13** and **14** with acid yielded the carboxylic acid (**15**) in a quantitative yield. The acid (**15**) was treated with methanol and ethanol in the presence of acid to afford the esters **13** and **14** in quantitative yields, respectively.





10, R=H
11, R=CH_3
12, R=CH_2CH_3

13, R=CO_2CH_3
14, R=$CO_2CH_2CH_3$
15, R=CO_2H
16, R=$CONHNH_2$
17, R=CON_3
18, R=$NHCO_2CH_2C_6H_5$.

The carboxylic acid (**15**) was submitted to Curtius rearrangement by the diphenylphosphinic azide method[10] to yield the desired 3-amino derivative (**7**), which was confirmed to be exactly identical with the product obtained by the modified Snyder's method.[11] In this method, either **13** or **14** was converted to the hydrazide (**16**), which was then treated with nitrous acid to afford the corresponding azide (**17**). The treatment of the azide (**17**) with benzyl alcohol in toluene gave the benzyl carbamate (**18**), which was further submitted to the acidic or alkaline hydrolysis to yield the amino compound (**7**). In comparison with the overall yields of **7** from **13**, this modified method was better than the diphenylphosphinic azide method. Recently, Agarwal *et al.*[12] reported that **7** could be synthesized starting from methyl DL-1,2,3,4-tetrahydro-9*H*-pyrido-[3,4-*b*]indole-3-carboxylate *via* a Curtius reaction. Their method is essentially the same as our modified method.

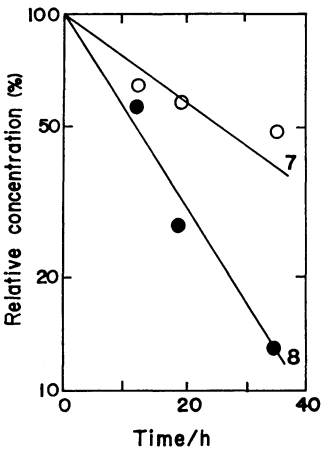


Fig. 5. Time course of degradation of **7** and **8**.

Experimental

Column chromatography was carried out over silica gel (Merck, Kieselgel 60). Merck pre-coated silica gel 60 F-254 TLC plates (5×20 cm²) and PLC plates (20×20 cm²) were used for the TLC and preparative TLC, respectively. Unless otherwise noted, the developing solvent system was a CH_2Cl_2–ethanol (9:1, v/v) mixture, and visualization was effected by the use of a Pan UV lamp. The homogeneity of each compound was always checked by TLC on silica gel. HPLC was made with a Toyo Soda Model HLC-803A equipped with an appropriate analytical column. Retention times of HPLC under various conditions are shown in Table 1. Absorption spectra were recorded with a Hitachi Model EPI-S_2 IR spectrophotometer and a Hitachi Model 124 spectrophotometer. Mass spectra were run with a Shimadzu LKB-9000 spectrometer, operating at an electron beam energy of 70 eV and with direct inlet. All melting points were taken on a hot-stage apparatus and are uncorrected.

Pyrolysis of L-Tryptophan. A hundred grams of L-tryptophan dried with P_2O_5 was pyrolyzed and dry-distilled by a direct flame at 400—500 °C for 1 h to give a tar (61 g). The tar was dissolved in ether (1 L) and the solution was extracted with 20% HCl (400 ml×5). The extracts were combined, cooled, and made alkaline with NaOH. The alkaline mixture was then extracted with ether (250 ml×6). The ethereal extract was washed (H_2O), dried (Na_2SO_4), and concentrated, giving 22.6 g of basic substances. The substances were dissolved in CH_2Cl_2 and fractionated by a silica-gel column (3×20 cm) using CH_2Cl_2 and CH_2Cl_2–EtOH (25:1 and 2:1, v/v) mixtures as elution solvents, giving three fractions (1st fr., 0.49 g; 2nd fr., 3.72 g; 3rd fr., 1.36 g). The second fraction was expected to contain 3-amino compounds, **7** and **8**, and was further fractionated on a column (5×20 cm) of silica gel into four subfractions by a CH_2Cl_2–EtOH (9:1, v/v) mixture to afford fractions, 2-

A-2-D. These procedures of fractionations are shown in Fig. 1. Each fraction was then subjected to preparative TLC to develop multiple times. In the cases of the lst fraction, fraction 2-C, and 3rd fraction, the number of times of development was three, and in the cases of fractions 2-A and 2-B it was two. In the case of 3rd fraction, the developing solvent system was a CH_2Cl_2–EtOH (4:1, v/v) mixture. A plate charged with fraction 2-D was developed six times.

The bands corresponding to **2**, **3**, **5**—**8**, and **9** were visualized under UV light. As shown in Fig. 1, band 1—1 was obtained from fr. 1, bands 3—1 and 3—2 from fr. 3, and band 2-D-1 from fr. D, respectively. Fractions 2-A, 2-B, and 2-C were each divided by similar chromatography into three bands. Each band was then scraped off, added to methanol, and mixed well. Each methanol mixture was centrifugalized and the resulting supernatant was then applied to HPLC to analyze the contents of the target compounds. These results are shown in Table 2.

*3-Amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (**2**).* Compound **2** was supplied under the Research Resources Program (No. 57010022) for Cancer Research, the Ministry of Education, Science, and Culture: TLC R_f 0.01; UV (MeOH) 241 (log ε 4.61), 263 (4.87), 303 (4.00), and 315 nm (4.00).

*2-Amino-9*H*-pyrido[2,3-*b*]indole (**3**).* Compound **3** was also supplied under the same program as described above: TLC R_f 0.47; UV (MeOH) 231 (log ε 4.10), 260 (3.66), and 337 nm (3.76).

*9*H*-Pyrido[3,4-*b*]indole (**5**).* Compound **5** was prepared by a procedure similar to that reported[13] and purified with column chromatography, giving needles (from dil MeOH): mp 199—200 °C; TLC R_f 0.30; UV (MeOH) 233 (log ε 4.61), 248 (sh, 4.37), 281 (sh, 4.09), 288 (4.29), 366 (3.67), and 349 nm (3.68); IR (KBr) 3260 and 1630 cm^{-1}.

*1-Methyl-9*H*-pyrido[3,4-*b*]indole (**6**).* Compound **6** was prepared as reported[14] and purified with the chromatography, giving needles (from dil MeOH): mp 229—230 °C; TLC R_f 0.22; UV (MeOH) 234 (log ε 4.63), 238 (sh, 4.62), 248 (4.43), 281 (sh, 4.07), 287 (4.29), 333 (3.75), and 347 nm (3.76); IR (KBr) 3210 and 1622 cm^{-1}.

*Compounds **11** and **12**.* To a suspension of **10** (8.8 g) in MeOH (200 ml), conc. H_2SO_4 (10 ml) was added. The mixture was refluxed for 8 h, concentrated, poured into ice, and made neutral with K_2CO_3. The resulting mixture was extracted with CH_2Cl_2 and the extract was then washed (H_2O), dried ($MgSO_4$), and concentrated, giving 7.6 g (81%) of the methyl ester (**11**): TLC R_f 0.69.

A mixture of **10** (13 g) in EtOH (200 ml) and conc. H_2SO_4 (12 ml) was treated as above to afford 7.78 g (54%) of the ethyl ester (**12**): TLC R_f 0.76.

*The Esters **13** and **14**.* *i) With Sulfur*: A suspension of **11** (2.6 g) and powdered sulfur (1 g) in xylene (40 ml) was refluxed for 6 h and cooled. The crystals which then formed were purified by column chromatography to yield 2.05 g (80%) of **13**. Recrystallization from EtOH gave needles: mp 255—257 °C (decomp); TLC R_f 0.48; UV (MeOH) 216, 232, 269, 301 sh, 330, 345 nm; IR (KBr) 3250, 1723, and 1710 cm^{-1}.

Found: C, 68.78; H, 4.35; N, 12.10%. Calcd for $C_{13}H_{10}N_2O_2$: C, 69.01; H, 4.46; N, 12.38%.

The ethyl ester (**14**) was prepared from **12** in a 68% yield under similar conditions to those described above: Needles (from C_6H_6–EtOH); mp 207—208 °C; TLC R_f 0.53; IR (KBr) 3250 and 1725 cm^{-1}.

Found: N, 11.35%. Calcd for $C_{14}H_{12}N_2O_2$: N, 11.66%.

The esters **13** and **14** were obtained in quantitative yield by the treatment of the acid (**15**) with MeOH and EtOH in the presence of H_2SO_4, respectively.

ii) With Lead(IV) Acetate: To a solution of **11** (2.3 g) in acetic acid (35 ml), lead(IV) acetate (9.3 g) freshly prepared from Pb_3O_4 was added, and the mixture was allowed to stand at room temperature for 30 min. After addition of ethylene glycol (1 ml), the mixture was poured into ice-water and made alkaline with dil. aqueous ammonia to yield 565 mg (25%) of **13**.

The ester **14** was obtained in a 28% yield by the same treatment of **12** with lead(IV) acetate.

*The Carboxylic Acid (**15**).* *i) From **13***: A suspension of **13** (1.0 g) in 5% aq NaOH solution (20 ml) was heated on a water bath for 8 h to give a clear solution. Acetic acid was added to the solution to make it acidic. The precipitate thereby formed was collected, washed (MeOH), and dried, giving the carboxylic acid (**15**) in a quantitative yield: mp 318—320 °C (decomp).

*ii) From **14***: Treatment of **14** under similar conditions gave **15** in a quantitative yield.

*3-Amino-9*H*-pyrido[3,4-*b*]indole (**7**).* *i) By Modified Snyder's Method*: To a suspension of methyl ester **13** (5 g) in 1-pentanol (50 ml) and ethanol (15 ml), 100% hydrazine hydrate (20 ml) was added. The mixture was refluxed for 5 h, cooled, and filtered, giving 4.92 g (98%) of the hydrazide (**16**): TLC R_f 0.06; IR (KBr) 3320, 3240 (broad), and 1650 cm^{-1}. To a solution of **16** (2.26 g, 10 mmol) in conc. HCl (1.5 ml) and water (100 ml), a solution of $NaNO_2$ (828 mg, 12 mmol) in water (5 ml) was added under cooling with ice-water. The resulting mixture was allowed to stand in the cold with stirring for 30 min and then neutralized with saturated $NaHCO_3$ solution to give 2.27 g (96%) of the azide (**17**): TLC R_f 0.55. A mixture of **17** (2.27 g) in xylene (25 ml; dried with "Dry Soda") and benzyl alcohol (2.2 ml; dried with molecular sieve 3A) was heated at 110—120 °C for 20 min and then allowed to stand in a refrigerator overnight to afford 2.81 g (92%) of the benzyl carbamate (**18**): mp 208—212 °C (decomp), TLC R_f 0.53. To a suspension of **18** (2.8 g) in ethylene glycol (30 ml) and water (3 ml), KOH (3 g) was added. The mixture was stirred and heated at 150—160 °C for 30 min to yield 1.40 g (86%) of the amine (**7**). The overall yield of **7** from **13** is 74%: Bright yellow scales (from aq DMF); mp 286—288 °C (blacken) and 290—291 °C (decomp); TLC R_f 0.11, R_f 0.14 with CH_2Cl_2–EtOH (4:1, v/v); UV (MeOH) 236 (log ε 4.53), 248 (sh, 4.49), 292 (sh, 4.00), and 299 nm (4.10); IR (KBr) 3460, 3420, 3300, and 1625 cm^{-1}.

Found: C, 72.05; H, 4.90; N, 22.71%; M^+ 183. Calcd for $C_{11}H_9N_3$: C, 72.11; H, 4.95; N, 22.94%; M 183.

The acidic hydrolysis of the carbamate (**18**) was made as follows: A suspension of **18** (159 mg, 5 mmol) in 10% HCl (10 ml) was refluxed for 5 h and then made alkaline with diluted KOH aq solution, giving 64 mg (70%) of **7**.

The ethyl ester **14** was converted into the hydrazide **16** in an 85% yield under similar conditions.

ii) By Diphenylphosphinic Azide Method: To a mixture of **15** (850 mg) in triethylamine (1 ml), diphenylphosphinic azide (1.20 g) was added. The resulting mixture was heated at 80 °C for 1 h. Benzyl alcohol (1 ml) was added to the mixture, which was further heated at 120 °C for 10 h. After purification with column chromatography, 150 mg (12%) of the carbamate **18** was obtained and the alkaline hydrolysis of **18** gave **7**. The overall yield of **7** from **15** is 10%.

*3-Amino-1-methyl-9*H*-pyrido[3,4-*b*]indole (**8**).* Compound **8** was prepared from the methyl ester by a procedure

of the method of Snyder *et al.*,[11] giving needles (from MeOH) (Found: C, 73.01; H, 5.59; N, 21.45%): mp 228—229 °C; TLC R_f 0.11; R_f 0.15 with CH_2Cl_2–EtOH (4:1, v/v); UV (MeOH) 240 (log ε 4.56), 291 (sh, 4.00), and 297 nm (4.09); IR (KBr) 3380, 3170 (broad), and 1632 cm^{-1}. MS M^+ 197.

*1-Amino-9H-pyrido[3,4-b]indole (**9**).* According to the method reported by Snyder *et al.*,[15] **9** was prepared by the reaction of **5** and $NaNH_2$ in liquid NH_3, giving needles (from dil EtOH) in a 95% yield: mp 203—204 °C (decomp), TLC R_f 0.03; R_f 0.19 with CH_2Cl_2–EtOH (4:1, v/v); UV (MeOH) 240 (log ε 4.70), 278 (3.90), 288 (4.00), 299 (3.47), 326 (3.73), 337 (3.85), and 350 nm (3.78); IR (KBr) 3420, 3370, 3310, and 1632 cm^{-1}.

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