Determination of Aspartic Acid, Phenylalanine, and Aspartylphenylalanine in Aspartame-Containing Samples Using a Precolumn Derivatization HPLC Method

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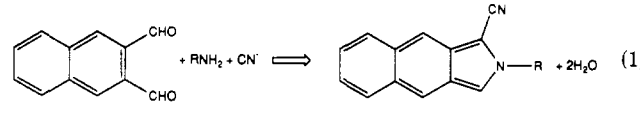
The sweetener aspartame (APM) and its hydrolysis products, aspartylphenylalanine (AP), aspartic acid (Asp), and phenylalanine (Phe), react with naphthalene-2,3-dicarboxaldehyde (NDA) in the presence of cyanide ion (CN) in a mildly alkaline medium to give highly fluorescent 1-cyano-2-substituted benz[*f*]isoindole (CBI) derivatives. These CBI derivatives are readily resolved by reverse-phase high-performance liquid chromatography, giving detection limits in the subpicomole range. Since APM can readily hydrolyze to AP or cyclize to give diketopiperazine (DKP) under alkaline conditions, accurate determination of the levels of AP in analytical samples is possible only after APM is separated from Asp, AP, and Phe by solid-phase extraction, followed by alkaline hydrolysis to give AP, which reacts quantitatively with NDA to form the fluorescent CBI product. Assay procedures are described for the determination of Asp, Phe, AP, and APM in diet beverage samples.

Routine quality assurance of food and pharmaceutical products requires analytical methods that can accurately determine the levels of the active ingredients, additives, and related degradation products. In food science research, it is well-known that the presence of small peptides in food substances can drastically alter the taste perception.

Aspartame (L-α-aspartyl-L-phenylalanine methyl ester, APM) is an effective sweetener currently used as a food additive and sugar substitute (Mazur et al., 1968; Mazur, 1976; Cloninger and Baldwin, 1970, 1974). The stability of APM is affected by temperature and moisture level and is highly dependent on the pH in aqueous solution (Homler, 1984). The degradation products of APM are L-α-aspartyl-L-phenylalanine (AP), derived from the hydrolysis of the methyl ester, and 2,5-disubstituted diketopiperazine (DKP), which is produced by the intramolecular aminolysis of the methyl ester linkage (Scheme I) (Holmer, 1984). Neither AP nor DKP is an effective sweetener. DKP can be converted to AP or L-phenylalanyl-L-aspartic acid (PA) by hydrolysis. Both AP and PA can be further hydrolyzed to give the individual amino acids aspartic acid (Asp) and phenylalanine (Phe).

High-performance liquid chromatography is an extremely useful method for the separation of amino acids and peptides, and many HPLC assays have been reported for the analysis of APM (Prudel and Davidkova, 1985; Tsang et al., 1985; Verzella and Mangia, 1985; Argoudelis, 1984; Daniels et al., 1984; Tyler, 1984; Webb and Beckman, 1984; Cross and Cunico, 1984; Fox et al., 1976; Furda et al., 1975; Hussein et al., 1984). However, the levels of Asp and Phe are generally not determined in these assays because, like most amino acids and peptides, Asp and Phe lack the physicochemical properties, such as low absorptivity, that would permit an accurate determination of their concentrations at the micromolar level.

The chromatographic detection sensitivity of these analytes can be enhanced by chemical derivatization (Sternson, 1981). Fluorogenic derivatizations specific for primary amines, such as the fluorescamine (Udenfriend et al., 1972) and *o*-phthalaldehyde–thiol (OPA–RSH) (Roth, 1971) methods, are useful derivatization techniques for the analysis of amino acids. A new primary amine specific fluorogenic reaction was recently reported which involves the reaction of the amine with naphthalene-2,3-dicarboxaldehyde (NDA) in the presence of cyanide ion (CN) (eq 1) (Matuszewski et al., 1987; de Montigny et



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al., 1987; Carlson et al., 1986; Roach and Harmony, 1987). The reaction affords highly fluorescent and stable 1-cyano-2-substituted benz[*f*]isoindoles (CBI). More importantly, CBI products obtained from the reaction of NDA with peptides such as AP and APM also exhibit high fluorescence quantum efficiency (Matuszewski et al., 1987; Carlson et al., 1986). This offers a significant advantage over the OPA–RSH reaction, which gives low fluorescence efficiency isoindoles with peptides and highly unstable isoindoles with many amines. The objective of this work is to determine the feasibility of developing an HPLC assay for the determination of Asp, Phe, AP, and APM in beverage samples using the new NDA–CN precolumn derivatization method.

EXPERIMENTAL PROCEDURES

Apparatus. The high-performance liquid chromatography solvent delivery system consisted of two Shimadzu (Columbia, MD) LC-6A liquid chromatograph pumps controlled by a Shi-

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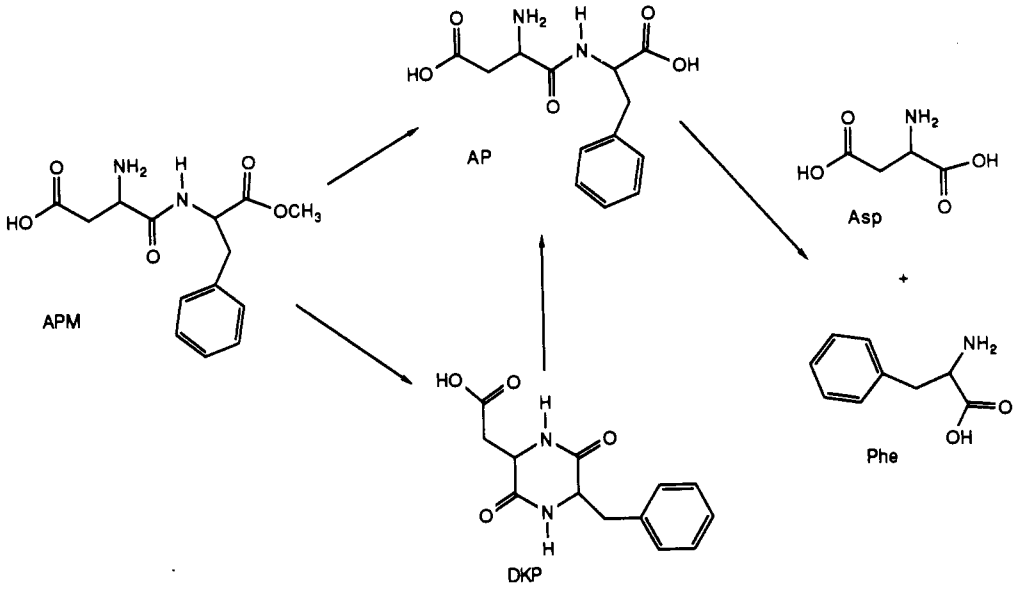
Scheme I. Aspartame and Degradation Products



madzu SCL-6A controller. The sample injections were performed by a Perkin-Elmer (Norwalk, CT) ISS-100 autoinjector. The detection system included a Shimadzu Model SPD-6AV absorbance detector set at 420 nm, using a tungsten light source and a Hitachi (Tokyo, Japan) F1000 fluorescence spectrophotometer, λ_{ex} = 420 nm and λ_{em} = 490 nm. Signals from these detectors were recorded by a dual-channel recorder and the relative peak areas recorded with a Perkin-Elmer LCI-100 laboratory computing integrator. All weight measurements were made on a Mettler analytical balance, and pH measurements were made by using an Orion digital pH meter. The prederivatization separations of the AP and APM in the diet samples were achieved by using Sep-Pak C18 cartridges from Waters Associates (Milford, MA). Separations of various CBI derivatives were carried out by using a TSK ODS-120T column (4.6 × 150 mm, Tosoh, Tokyo, Japan).

Chemicals. Acetonitrile (HPLC) grade), methanol (ACS grade), sodium tetraborate (ACS grade), sodium cyanide (ACS grade), sodium acetate (anhydrous, ACS grade), and boric acid (ACS grade) were used as supplied. Asp, Phe, AP, and APM were all purchased from Sigma Chemical Co. (St. Louis, MO). The aldehyde NDA was prepared in Professor Robert Carlson's laboratory at the University of Kansas. The water used was purified by using a four-module Sybron/Barnstead (Boston, MA) Nanopure II system and was stored in glass for no longer than 5 days.

Procedure. *Derivatization Procedure.* Typically, 0.10 mL of an analyte solution ([amine] = 10 μM) was added to 0.76 mL of borate buffer (50 mM, pH 8.0). Then, 0.10 mL of 10 mM NaCN (in water) was added and mixed. The derivatization reaction was initiated by the addition of 40 μL of 5 mM NDA (dissolved in MeOH). Unless otherwise stated, the final concentrations were NDA, 0.2 mM, and NaCN, 1.0 mM. The mixture was protected from light and incubated at room temperature for 2 h. Sodium cyanide and potassium cyanide are highly toxic chemicals; therefore, it is important to prepare and handle these reagents and solutions carefully.

HPLC Separation of CBI Products. The two eluents used for the gradient separations were (A) 50 mM acetate buffer, pH 6.0, and (B) 90% acetonitrile in water. CBI derivatives of APM and its hydrolysis products were separated by using the following gradient method: 0–3 min, 30% B; 3–4 min, 45% B; 4–14 min, 45% B; 14–15 min, 80% B; 15–18 min, 80% B; 18–19 min, 30% B.

Detection Response of Amine Standards. The standard curves for AP, APM, Asp, and Phe were determined by the following procedure. Four to five concentrations of each amine, ranging between 0.02 and 10.0 μM, were derivatized following the procedure previously described. Ten microliters of the reaction solution was injected for each amine concentration, i.e., 0.2–100 pmol was analyzed by HPLC. The area units were then plotted against picomoles injected to determine the standard slope of area units per picomole injected.

Stability of APM and AP in Borate Buffer Reaction Solution. Aliquots of APM dissolved in borate buffer (50 mM, pH 8.0) were withdrawn at different time intervals (0, 60, and 120 min), derivatized as described above, and analyzed by HPLC. The sampling time of each aliquot was plotted against the peak areas obtained for APM and AP. The same procedure was used to determine the stability of AP in pH 8 borate buffer.

Hydrolysis Rates of APM in Various NaOH Solutions. One hundred microliters of 1.0 mM APM was added to each test tube containing 10 mL of different concentrations (1.0, 10, and 100 mM) of sodium hydroxide (NaOH). After mixing, the solution was incubated at room temperature (25 °C). At appropriate time intervals, 100 μL of each solution was derivatized and analyzed by HPLC following the procedures described above.

Stability of AP in NaOH. AP was added to a solution of 0.1 N NaOH to give a final concentration of 100 μM in AP. After 30 min, 1.0 mL of the reaction solution was diluted 10 times with borate buffer (pH 8.0, 50 mM). This diluted solution was derivatized and analyzed by HPLC.

Efficiency of Alkaline Hydrolysis of APM to AP. Sodium hydroxide was added to an APM solution to give a 14.5 μM solution of APM at room temperature. After 20 min, 100 μL of APM solution was derivatized. The amount of AP–CBI was analyzed by HPLC after the derivatization reaction was completed.

Analysis of APM and AP in Liquid Diet Samples. a. Preparation of Sample. The diet sample was degassed by stirring for 20 min. Three milliliters of 10 mM acetate buffer (pH 5.0) was added to 2 mL of the degassed diet sample. Standard AP (50 μM) and APM (1.0 mM) samples were prepared similarly.

b. Separation of APM and AP. The C18 cartridges were activated by sequential elution with 10 mL of methanol, 2 mL of 10% methanol in sodium acetate buffer (10 mM, pH 5), and 5 mL of acetate buffer. After the cartridge was prepared, 1.0 mL of the prepared sample was loaded onto the Sep-Pak, followed by (1) 3.0 mL of 5% methanol in the acetate buffer, (2) 3.0 mL of 10% methanol in acetate, and (3) 6.0 mL of 30% methanol in acetate. Since it was found that the Asp and AP were completely eluted from the Sep-Pak in the 5% and 10% methanol fractions, three collection vials were used. Fractions from the sample and 5% methanol elutions were combined in vial 1, while the 10% methanol elution was collected in vial 2, and the 30% elution was collected in vial 3. Because each batch of solid-phase cartridges may give different performance, it was important to calibrate the elution profile of a new lot of cartridge by using a relevant standard amine, such as Asp, before the method was applied to the samples.

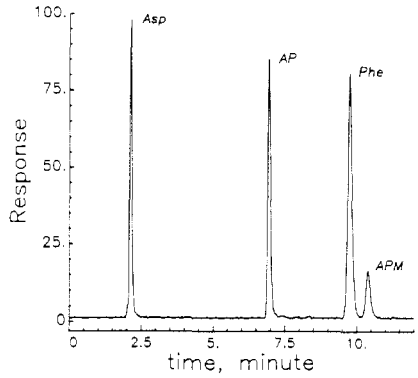


Figure 1. Reverse-phase chromatographic separation of CBI products of AP, APM, Asp, and Phe, mobile phase A (50 mM NaOAc, pH 6.0) and B (90% acetonitrile, 10% water). The gradient profile used is described under Experimental Procedures. Amine concentrations were 10 μM each, and the injection volume was 10 μL.

c. Hydrolysis of APM. One milliliter of eluent from the third vial containing APM was diluted with 9.0 mL of 0.1 N NaOH and hydrolyzed for 20 min. Unless stated otherwise, this was the standard method for hydrolysis.

d. Derivatization Procedure. The same derivatization procedure as previously described was utilized. A 100 mM borate buffer was used instead of 50 mM.

Recovery of AP and APM from Solid-Phase Extraction. APM (1.47 mM, 2.0 mL) was diluted to 10 mL with 10 mM sodium acetate, pH 5. After 1.0 mL of this solution was loaded onto the Sep-Pak cartridge, it was eluted in the same manner as described above in section b. The 30% methanol elution was hydrolyzed as before. After 20 min, 0.10 mL of the hydrolysis solution was derivatized as before. For AP, 2.0 mL of a 50 μM solution of AP in sodium acetate was loaded onto the Sep-Pak and eluted as described above. The three fractions were then derivatized and analyzed by HPLC.

RESULTS AND DISCUSSION

Cyanobenz[*f*]isoindole Derivatives of AP, APM, Asp, and Phe. Figure 1 shows the separation of a mixture of CBI derivatives of AP, APM, Asp, and Phe using RP-HPLC with fluorescence detection [420 nm (ex), 490 nm (em)]. The four analytes were readily resolved within 12 min by using an acetonitrile gradient. A plot of the integrated peak area versus amount of CBI injected for each analyte showed good linearity within the range (0.2–100 pmol) examined, and subpicomole detection limits (≈0.1 pmol) were realized for the four analytes ($S/N \geq 3$).

If the analytes (Asp, AP, APM, and Phe) were quantitatively converted to the CBI products, the slope of the line for each amine was related to the fluorescence quantum efficiency of the CBI product under these chromatographic conditions. For APM–CBI, the slope in units of area unit per picomole was found to be approximately 3 times smaller than that of AP–CBI.

Careful examination of the factors affecting the yields of the CBI derivatives of these amines showed that the yield of the APM–CBI, unlike that of other amino acids and peptides, was highly sensitive to pH, buffer concentration, and reaction temperature. The calibration results described above were obtained under optimal conditions found for the simultaneous derivatization of AP, Asp, and Phe. Under these reaction conditions, the yield of the APM–CBI product was lower than that of the other peptide, AP. Increasing the borate buffer concentration to 0.75 M and reducing the reaction temperature to 4 °C increased the yield of APM–CBI 2-fold. Under these conditions, the yield of APM–CBI was found to be highly reproducible. It is suspected that the low yield obtained under standard conditions was due to the competitive formation of DKP.

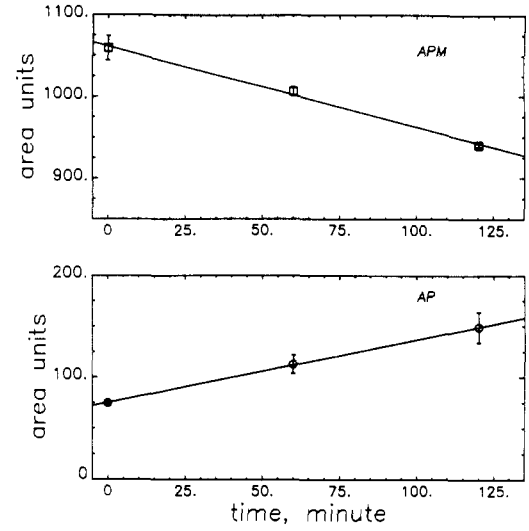


Figure 2. Stability of APM in alkaline borate buffer reaction solution. APM and AP were determined as the respective CBI products. (A) APM; (B) AP.

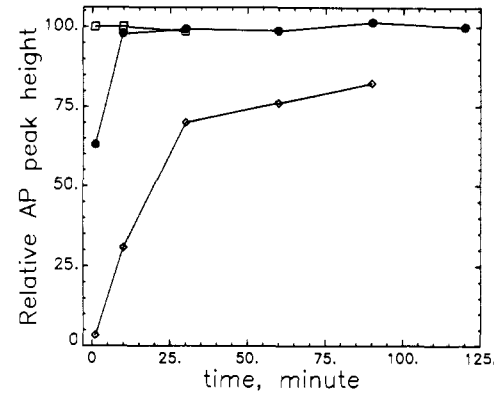


Figure 3. Hydrolysis of APM to give AP in sodium hydroxide solutions. AP was determined as the CBI product by using HPLC; [NaOH] = 1 (◇), 10 (●), and 100 mM (□).

Stability of APM and AP in Alkaline Borate Buffer. In aqueous solution, APM is reported to be stable within the pH range 3–5. Above pH 6, rapid hydrolysis of the methyl ester function of APM to give AP was reported (Holmer, 1984). Figure 2 shows the stability of APM in pH 8 borate buffer (50 mM) over a 120-min period. A significant decrease in the APM peak area was observed with a concomitant increase in the peak area of AP, indicating the hydrolysis of APM to AP under these reaction conditions. The estimated hydrolysis rate of 29 h^{-1} recorded here at pH 8 was consistent with reported values (Holmer, 1984). Similar experiments with AP in borate buffer (pH 8) showed no change in the peak area of AP, which is reasonable since (1) the amide linkages of peptides are known to be more stable than ester bonds and (2) the cyclization of AP to DKP is not favorable under these conditions. The optimal derivatization conditions for AP, Asp, and Phe require a slightly alkaline aqueous medium, pH 8.0–9.0. The instability of APM under such reaction conditions renders impractical the simultaneous determination of AP and APM by the NDA derivatization method.

Hydrolysis of APM and Stability of AP in Sodium Hydroxide. Figure 3 shows the time profile of hydrolysis of APM to AP in various concentrations of NaOH (1, 10, and 100 mM). Hydrolysis of APM was rapid, and

Table I. Alkaline Hydrolysis Efficiency of APM To Give AP[a]

observed hydrolysis	peak area ± SD
APM in 0.10 NaOH	5588 ± 23
predicted from AP standards	5582 ± 55
hydrolysis efficiency, %	100.1 ± 1.1

[a] Peak area of AP measured as AP-CBI product.

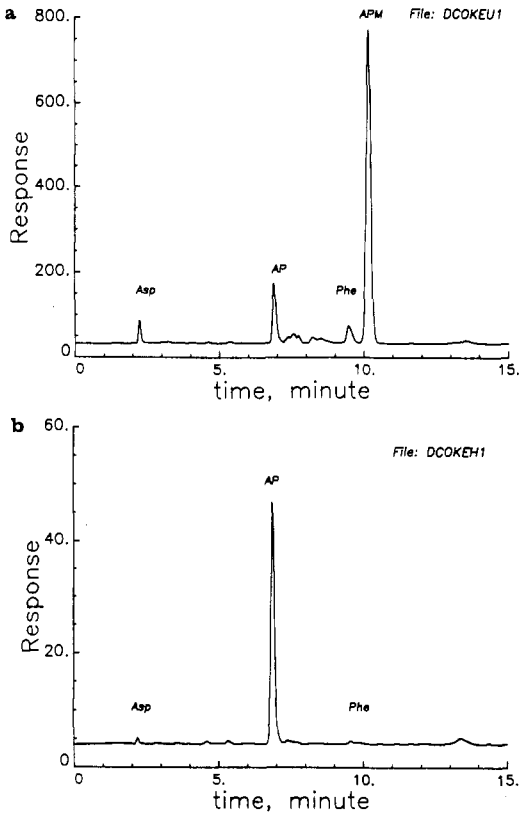


Figure 4. Chromatograms of a diet sample before and after hydrolysis. (a) Sample was derivatized with NDA after sample was diluted 10 times with 10 mM, pH 5, sodium acetate, giving an overall dilution factor of 100. (b) Sample was hydrolyzed with 0.1 M NaOH before derivatization (total dilution is 1000 times).

complete conversion (see Table I) of APM to AP was observed in less than 20 min at room temperature in 100 mM NaOH. More importantly, the data showed that (1) AP was stable in the hydrolysis medium and (2) the formation of DKP could be ignored under these reaction conditions (see Table I). An accurate determination of AP and APM was possible if AP in the analytical sample was separated from APM prior to derivatization. APM could then be quantified as the fluorescent CBI product after alkaline hydrolysis to give AP, followed by derivatization with NDA.

Determination of Asp and Phe in Diet Beverage Samples. Figure 4a shows a chromatogram of a diet sample that was diluted with sodium acetate buffer, followed by derivatization with NDA. Peaks associated with the four analytes were detected, and the concentrations of Asp and Phe in the sample were determined directly. The spectroscopic properties of these analytes, especially Asp, which has poor absorbance characteristics, were not amenable to absorptive detection at low and submicromolar concentrations. The described derivatization method permitted the simultaneous determination of these analytes (Asp and Phe) at subpicomole levels.

Determination of AP and APM. Figure 4b shows a chromatogram of the same diet sample following identical treatment, except that the mixture was hydrolyzed with 0.10 M NaOH before derivatization. This procedure was used to determine the total AP equivalence (AP + APM) present in the sample.

Table II. Separation and Recovery of AP and APM[a]

fraction	elution condition	% recovery AP	% recovery APM
1	1.0 mL of sample + 3.0 mL of 5% MeOH in 5 mM acetate (pH 5)	97.0	0
2	3.0 mL of 10% MeOH in acetate buffer (pH 5)	0	0
3	6.0 mL of 30% MeOH	0	98.6
	total recovery	97.0 ± 2.6	98.6 ± 1.8

[a] $n = 3$.

Table III. Summary of Diet Sample Results

sample (n)[a]	Asp ± SD, μM	AP ± SD, μM	Phe ± SD, μM	APM ± SD, mM
A (1)	22.9	58.8	25.2	1.68
B (2)	31.5 ± 0.1	60.7 ± 1.0	42.3 ± 1.3	1.50 ± 0.06
C (3)	25.7 ± 1.4	68.4 ± 1.3	30.9 ± 1.9	1.60 ± 0.04
C (1)	27.0	70.5	29.2	1.64

[a] n = number of runs.

Accurate measurement of AP and APM concentrations was possible after the two analytes in the sample were separated by solid-phase extraction. The elution pattern and recovery of a standard mixture of AP and APM obtained by using this procedure are summarized in Table II. Table III lists the results obtained from the analysis of three different diet beverage samples by using the above procedures. The concentrations of Asp and Phe were very similar in each sample, ranging from 23 to 42 μM. This suggested that these two analytes may have been generated from the slow hydrolysis of AP and/or APM. The levels of AP and APM found were 59–71 μM and 1.50–1.64 mM, respectively. Sample C was analyzed over a 2-day period. A single determination on the second day gave results consistent with data recorded in the first day from multiple determinations. The mean coefficient of variation of this analytical procedure for the four analytes was 3%.

Several assays are available for the determination of APM, DKP, and AP in food preparations using reverse-phase and ion-exchange separation methods with absorbance or fluorescence detection, which give detection limits in the 100-pmol range. The concentrations of APM in beverages are usually in the millimolar range. Therefore, by use of a 10-μL sample loop, nanomole quantities of APM are typically injected. As such, detection sensitivity is not a problem. However, the concentrations of Phe and, especially, Asp, being in the micromolar range, are usually not determined because of insufficient sensitivity. The assay method described in this work provides improved sensitivity, enabling the detection of AP, Asp, and Phe in the sample, although the peptide and amino acids are 2 orders of magnitude lower in concentration than those of APM. In summary, our results show that the NDA–CN derivatization method is useful for the sensitive determination of Asp, Phe, and AP in beverage preparations.

ACKNOWLEDGMENT

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Registry No. APM, 22839-47-0; AP, 13433-09-5; Asp, 56-84-8; Phe, 63-91-2.

Synthesis and Herbicidal Activity of the Halo Analogues of Pyoluteorin

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The synthesis of some halo analogues of pyoluteorin [2,3-dichloro-5-(2′,6′-dihydroxybenzoyl)-1*H*-pyrrole] through the use of a Friedel–Crafts aroylation of pyrrole with 2,6-dimethoxybenzoyl chloride, followed by halogenation and demethylation with boron tribromide, is described. Stepwise bromination of the intermediate 2-(2′,6′-dimethoxybenzoyl)pyrrole yielded the di-, tri-, and tetrabromo derivatives. Similarly, chlorination gave the dichloro and the trichloro derivatives whereas iodination with even an excess of iodine gave only the monoiodo derivative. A comparison of the herbicidal activity using a cress seedling assay indicated that some of the halo analogues were 2–40 times as active as the parent compound, pyoluterin, with the tribromo analogue being the most active. This activity range appears to be comparable to that seen with some of the commercially used herbicides tested in this assay.

Since the discovery of the antibiotic pyoluteorin (1) isolated from a *Pseudomonas spp.* by Takeda (1958a,b, 1959), a number of syntheses have been described (Elix and Sargent, 1967; Bailey and Rees, 1970; Birchall et al.,

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