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AQUEOUS PHOTOLYSIS OF ^{14}C-BAS 654 H UNDER ARTIFICIAL SUNLIGHT

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Abstract - Photolysis of ^{14}C-BAS 654 H (pyridine and phenyl labels) was conducted in aqueous buffers of pH 5, 7 and 9 at 22 ± 1° C under sterile conditions using artificial sun light source. BAS 654 H degraded photolytically to a number of products as shown in Scheme 3. Identity of the photo degradation products was established employing HPLC co-chromatography with reference standards and/or spectral data (MS and ^{1}H-NMR).

INTRODUCTION AND RESULTS SECTION

BAS 654 H (**1**) is an auxin transport inhibitor and has been developed by BASF Corporation for use as a post-emergent herbicide for the control of a wide range of broad-leaf weeds and grasses in corn. Photolysis of radiolabeled BAS 654 H (**1**) in aqueous buffers of pH 5, 7 and 9 under artificial sunlight was conducted to fulfill a requirement for the registration and sale of the product. The experimental procedures and the results for the study are presented herein.

Material Balance - The average material balance for pyridine labeled ^{14}C-BAS 654 H samples photolyzed in pH 5, 7 and 9 buffers was 98%, 102% and 103% of the total applied radioactivity (TAR) respectively. The average material balance for phenyl labeled ^{14}C-BAS 654 H samples photolyzed in pH 5, 7 and 9 buffers was 94%, 97% and 98% TAR respectively. For the dark controls (non-photolyzed) the average material balance ranged between 99-102% TAR and 98-101% TAR for the pyridine and phenyl label samples respectively. Material balance was the sum of the radioactivity found in buffer solution and volatile radioactivity found in NaOH trap. No effort was made to collect volatile radioactivity in the dark control samples.

Approximately 1% (pyridine label) and 7-17% (phenyl label) of the applied radioactivity was found as volatile. All of the volatile radioactivity was found in NaOH trap. Essentially all of the volatile radioactivity was identified as $^{14}CO_2$.

Distribution of Radioactive Products (Pyridine Label, Irradiated)

pH 5 Buffer - The HPLC analysis results revealed a total of 18 radioactive products (P1: t_R = 5.20-6.20; P2: t_R = 7.90-8.80; P3: t_R = 8.80-9.30; P4: t_R = 9.30-10.30; P5: t_R = 10.30-10.90; P6: t_R = 10.90-11.50; P7: t_R = 11.50-12.30; M6: t_R = 12.0-13.5; M1: t_R = 13.20-14.80; M24: t_R = 14.40-15.20; P8: t_R = 16.50-18.0; P9: t_R = 19.0-19.70; P10: t_R = 20.70-21.0; P11: t_R = 21.0-21.60; BAS 654 H: t_R = 22.60-24.50; M23: t_R = 24.30-25.60; P12: 25.60-26.10; M5: t_R = 26.10-26.90. BAS 654 H and M6 were found to be the major radioactive products at every sampling interval. The amount of BAS 654 H decreased with time and ranged between 97-26 %TAR during the course

of the study. The degradation product M6 increased over time and accumulated to 41.0% TAR at about 690 h. The other significant degradation products were M1, M23 and M24 and accumulated to a maximum of 4%, 5% and 7% TAR respectively. The rest of the degradation products were minor and were less than 4% TAR.

pH 7 Buffer - The HPLC analysis results revealed a total of 16 radioactive products (P1: t_R = 5.20-6.20; P2: t_R = 7.90-8.80; P4: t_R = 9.30-10.30; P5: t_R = 10.30-10.90; P6: t_R = 10.90-11.50; P7: t_R = 11.50-12.30; M6: t_R = 12.0-13.5; M1: t_R = 13.20-14.80; M24: t_R = 14.40-15.20; P9: t_R = 19.0-19.70; P10: t_R = 20.70-21.0; P11: t_R = 21.0-21.60; BAS 654 H: t_R = 22.60-24.50; M23: t_R = 24.30-25.60; P12: t_R = 25.60-26.10; M5: t_R = 26.10-26.90. BAS 654 H, M6 and M24 were found to be the major radioactive products. The amount of BAS 654 H decreased with time and ranged between 97 – 30 %TAR during the course of the study. The degradation product M6 increased over time and accumulated to 33% TAR after about 476 h of irradiation. The degradation product M24 increased to 17% TAR during the course of the study. The other significant degradation products were M1, P11 and M23 and accumulated to a maximum of 4%, 6% and 9% TAR respectively. The rest of the degradation products were minor and were below 2% TAR.

pH 9 Buffer - The HPLC analysis results revealed a total of 14 radioactive products (P1: t_R = 5.20-6.20; P2: t_R = 7.90-8.80; P3: t_R = 8.80-9.30; P5: t_R = 10.30-10.90; P7: t_R = 11.50-12.30; M6: t_R = 12.0-13.5; M1: t_R = 13.20-14.80; M24: t_R = 14.40-15.20; P8: t_R = 16.50-18.0; P9: t_R = 19.0-19.70; P10: t_R = 20.70-21.0; P11: t_R = 21.0-21.60; BAS 654 H: t_R = 22.60-24.50; M5: t_R = 26.10-26.90. BAS 654 H, M1, M6, P2 and P5 were found to be the major radioactive products. The amount of BAS 654 H decreased with time and ranged between 96 – 55 %TAR during the course of the study. The degradation products M1, M6, P2 and P5 increased over time to 9%, 10%, 7% and 13% TAR respectively after about 476 h of irradiation. The rest of the degradation products were minor and were below 3% TAR.

Distribution of Radioactive Products (Phenyl Label, Irradiated)

pH 5 Buffer - The HPLC analysis results revealed a total of 10 radioactive products (P1: t_R = 4.20-7.0; P2: t_R = 6.80-7.50; P3: t_R = 7.70-9.30; P4: t_R = 9.30-10.80; M7: t_R = 15.10-16.80; M2: t_R = 18.30-20.90; P11: t_R = 20.90-22.60; BAS 654 H: t_R = 22.50-24.70; M23: t_R = 24.50-26.10; M5: t_R = 26.30-27.50. BAS 654 H, M2, P11 and M23 were found to be the major radioactive products at every sampling interval. The amount of BAS 654 H decreased with time and ranged between 95 – 36 %TAR during the course of the study. The maximum concentration of M2 was 19% TAR and declined with time to 9% TAR. The degradation products P11 and M23 accumulated to 7% TAR and 9% TAR respectively after about 570 h of irradiation. The other significant degradation product was P4 which increased to 5% TAR at end of the study. The remaining degradation products were minor and were less than 4% TAR.

pH 7 Buffer -. The HPLC analysis results revealed a total of 9 radioactive products (P1: t_R = 4.20-7.0; P4: t_R = 9.30-10.80; M7: t_R = 15.10-16.80; P9: t_R = 17.90-19.90; M2: t_R = 18.30-20.90; P11: t_R = 20.90-22.60; BAS 654 H: t_R = 22.50-24.70; M23: t_R = 24.50-26.10; M5: t_R = 26.30-27.50. BAS 654 H, M2, P9 and P11 were found to be the major radioactive products at every sampling interval. The amount of BAS 654 H decreased with time and ranged between 95 – 46 %TAR during the course of the study. The maximum concentration of M2 was 11% TAR and declined with time to 8% TAR. The degradation products P9 and P11 increased with time to 13% TAR and 5% TAR respectively after about 642 h of irradiation. The rest of the degradation products were minor and were less than 4.8% TAR.

pH 9 Buffer - The HPLC analysis results revealed a total of 9 radioactive products (P1: t_R = 4.20-7.0; P3: t_R = 7.70-9.30; M7: t_R = 15.10-16.80; P8: t_R = 16.80-17.80; P9: t_R = 17.90-19.90; M2: t_R =

18.30-20.90; P11: t_R = 20.90-22.60; BAS 654 H: t_R = 22.50-24.70; M5: t_R = 26.30-27.50. BAS 654 H, M7, P1 and P9 were found to be the major radioactive products at every sampling interval. The amount of BAS 654 H decreased with time and ranged between 95 – 37 %TAR during the course of the study. The degradation products M7, P1 and P9 increased with time to 6% TAR, 10% TAR and 34% TAR respectively after about 642 h of irradiation. The rest of the degradation products were minor and were less than 2% TAR.

Distribution of Radioactive Products (Pyridine Label, Dark Control)

pH 5 Buffer - The HPLC analysis results revealed a total of 7 radioactive products P5, M6, M1, P9, BAS 654 H, M23 and M5. BAS 654 H, M1 and M6 were found to be the major radioactive products at every sampling interval. The amount of BAS 654 H decreased with time to 44%TAR during the course of the study. The degradation products M1 and M6 increased over time to 43% TAR and 8% TAR respectively. The rest of the degradation products were minor and were less than 2% TAR.

pH 7 Buffer - The HPLC analysis results revealed a total of 10 radioactive products P6, P7, M6, M1, P9, P10, P11, BAS 654 H, M23 and M5. BAS 654 H and M1 were found to be the major radioactive products at every sampling interval. The amount of BAS 654 H decreased with time to 72%TAR during the course of the study. The degradation product M1 increased over time to 28% TAR. The rest of the degradation products were minor and were less than 2% TAR.

pH 9 Buffer - The HPLC analysis results revealed a total of 9 radioactive products P5, P7, M6, M1, P9, P10, P11, BAS 654 H and M5. BAS 654 H and M1 were found to be the major radioactive products at every sampling interval. The amount of BAS 654 H decreased with time to 73 %TAR during the course of the study. The degradation product M1 increased over time to 28% TAR. The rest of the degradation products were minor and were less than 1% TAR.

Distribution of Radioactive Products (Phenyl Label, Dark Control)

pH 5 Buffer - The HPLC analysis results revealed a total of 6 radioactive products M7, P9, M2, P11, BAS 654 H and M5. BAS 654 H, M2 and M7 were found to be the major radioactive products at every sampling interval. The amount of BAS 654 H decreased with time to 43%TAR during the course of the study. The degradation products M2 and M7 increased over time to 47% TAR and 5% TAR respectively. The rest of the degradation products were minor and were less than 3% TAR.

pH 7 Buffer -. The HPLC analysis results revealed a total of 7 radioactive products P1, P9, M2, P11, BAS 654 H, M23 and M5. BAS 654 H, P9 and M2 were found to be the major radioactive products at every sampling interval. The amount of BAS 654 H decreased with time to 55%TAR during the course of the study. The degradation products P9 and M2 increased over time to 11% TAR and 34% TAR respectively. The rest of the degradation products were minor and were less than 3% TAR.

pH 9 Buffer - The HPLC analysis results revealed a total of 6 radioactive products P8, P9, M2, P11, BAS 654 H and M5. BAS 654 H, P9 and M2 were found to be the major radioactive products at every sampling interval. The amount of BAS 654 H decreased with time to 57%TAR during the course of the study. The degradation products P9 and M2 increased over time to 30% TAR and 13% TAR respectively. The rest of the degradation products were minor and were less than 2% TAR.

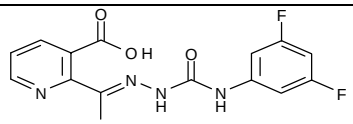
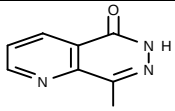
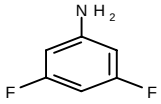
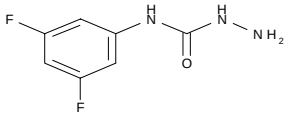
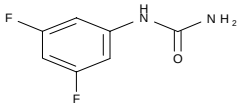
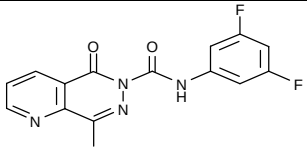
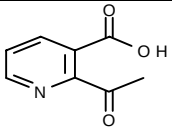
Half-Life

The photolysis half-lives for BAS 654 H in pH 5, 7 and 9 test buffers were 16 days, 27 days and 22 days respectively (mean of pyridine and phenyl). The dark control half-lives for BAS 654 H in pH 5, 7 and 9 test buffers were 23 days, 39 days and 41 days respectively (mean of pyridine and phenyl).

Identification of Photolysis Products

The identity of the isolated photodegradation products was established with a combination of HPLC co-chromatography with reference standards, if available, and/or MS spectrometry. The chemical structure and HPLC retention time of the reference standards are given in Table I.

Table I. Chemical Name, HPLC Retention Time and Structure of Reference Standards.

Product Identification Code and t_R (min)	Structure
BAS 654 H ~23.8	
M1 ~13.6	
M2 ~21.5	
M7 ~16	
M4 ~19.9	
M5 ~27	
M6 ~12.7	

HPLC Co-chromatography

There is a lag time of about a 10 - 20 sec between UV- and ^{14}C - responses for BAS 654 H with the UV signal appearing first. This was determined by making a single injection of a premixed BAS 654 H (non-labeled reference standard) and ^{14}C - BAS 654 H. The co-injection chromatograms for the isolated products showed the similar lag time difference between the UV -

and the ^{14}C - signals as observed in the experimental determination of lag time. These results indicate that the isolated radioactive products are the same compounds as the reference standards.

MS Spectrometry

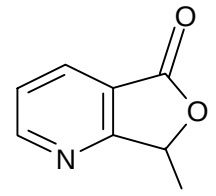
The radioactive products isolated from the aqueous photolysis experiments and the available reference standards were analyzed by mass spectrometry (LC-MS/MS positive ion mode and negative ion mode or GC-MS). Multiple Reaction Monitoring (MRM) for LC-MS/MS and Selective Ion Monitoring (SIM) for GC-MS techniques were utilized for MS spectrometric confirmation. The MS spectrum analysis data are listed in Tables II - IV.

Table II. MS Spectral Analysis Data for Reference Standards.

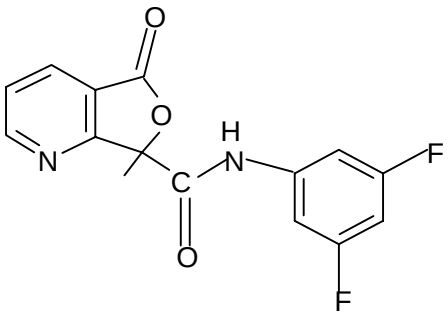
Code (Nominal Mol. Weight)	Observed m/z	MS Technique
BAS 654 H (334)	335 to 206, 335 to 162	LC-MS/MS
M1 (161)	162 to 93, 162 to 76	LC-MS/MS
M2 (129)	129, 102, 101	GC-MS
M4 (172)	173 to 130, 173 to 110	LC-MS/MS
M5 (316)	317 to 162, 317 to 103	LC-MS/MS
M6 (165)	166 to 148, 166 to 120	LC-MS/MS
M7 (188)	188 to 130, 188 to 110	LC-MS/MS

Table III. MS Spectral Analysis Data for ^{14}C-Products Isolated from Aqueous Photolysis Experiments.

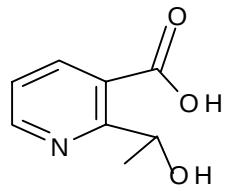
Pyridine Label	Observed m/z	Product Code (Structure)
HPLC Peak ~13 min	166 to 148, 166 to 120	M6 (see Table I)
HPLC Peak ~14 min	162 to 93, 162 to 76	M1 (see Table I)
HPLC Peak ~15 min	149/151,134/136, 106/108, 78/80	M24 (see below)



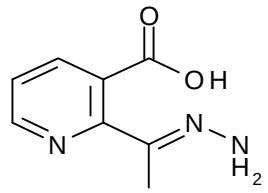
HPLC Peak ~24 min	335 to 206, 335 to 162	BAS 654 H(see Table I)
HPLC Peak ~25 min	305/307, 287/289, 148/150, 120/122	M23 (see below)



HPLC Peak ~8 min (P2) M25 (see below)



HPLC Peak ~10 min (P5) (see below)



+

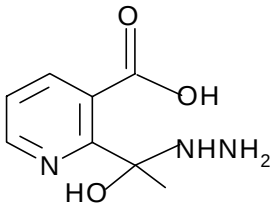
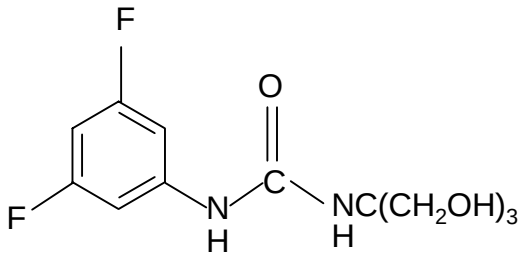
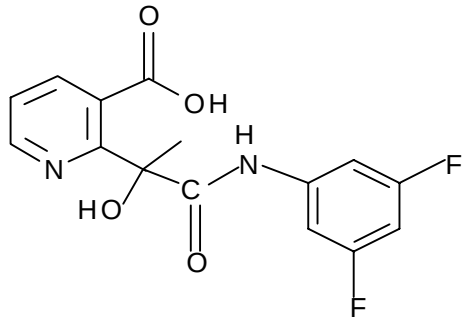


Table IV. MS Spectral Analysis Data for ^{14}C-Products Isolated from Aqueous Photolysis Experiments.

Phenyl Label	Observed m/z	Product Code (Structure)
HPLC Peak ~16 min	188 to 130, 188 to 110	M7 (see Table I)
HPLC Peak ~21 min	129, 102, 101	M2 (see Table I)
HPLC Peak ~19 min (P9)	277, 259, 122(TRIS), 104	(see below)



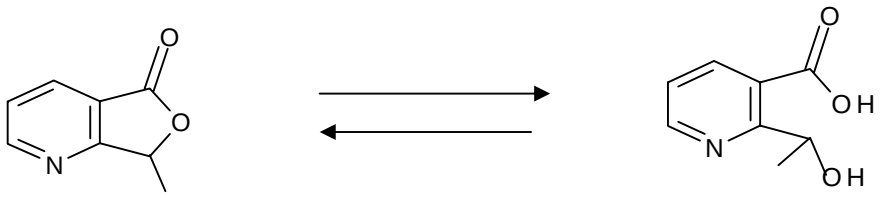
HPLC Peak ~19 min (minor product)	173 to 130, 173 to 110	M4 (see Table I)
HPLC Peak ~21.5 min (P11)	323 to 305, 325 to 307	(see below)



MS spectral analysis results for the isolated products are in complete agreement with the structures assigned to the isolated products.

Identification of the Isolated polar HPLC peak ~8-9 min (P2) – The structure of this peak was assigned as M25 and is shown in the Scheme 1.

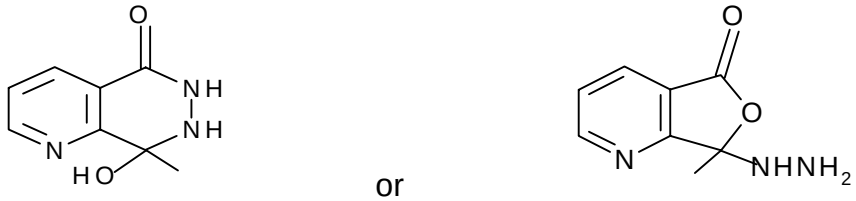
Scheme 1



M24 M25

The structure assignment is based on its chromatographic behavior and spectroscopic data for its transformation product (M24). The products M25 and M24 exist in equilibrium at neutral pH. A pH 9 buffer will contain mostly M25, whereas in pH 5, M24 will be the dominant product. By the time we processed our HPLC fractions corresponding to HPLC peak ~8-9 min for chromatographic analysis, the sample containing M25 had already changed to M24. This was because our HPLC mobile phase B contained 0.1% TFA. This was also confirmed by LC/MS analysis of the isolated sample. The identity of this ~8-9 min peak was further established by taking an aliquot of the positively identified ^{14}C-M24 (~15 min peak, isolated from pyridine label pH 5 experiment) and treating it with a catalytic amount of 0.1N NaOH. M24 changed into a product with same retention as M25 (P2) after the base treatment.

Identity of the Degradation Product P5 (~9-10 min HPLC Peak) - The degradation product P5 after isolation had transformed to 2 components (see the isolation, EXPERIMENTAL). These 2 components were isolated and analyzed by HPLC, LC/MS and GC/MS. The more polar component was confirmed as M1. Based on the mass spectral data (m/z:179 [M^+], 161 [M^+-H_2O, base peak], 146 [M^+-CH_5O], 132, 118, 104. ^{14}C – isotopic masses were also observed for these masses in the same ratio as found in ^{14}C - BAS 654 H. A proposed structure for the less polar component is shown below.



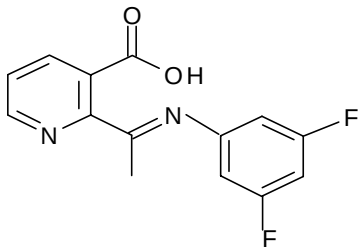
or

Based on this information, the possible structures of the precursors for the 2 components present in the degradation product P5 are shown below.

A and B

Most likely, during the isolation process product A, shown above transformed to M1 by loss of a water molecule and product B, shown above changed to the other component.

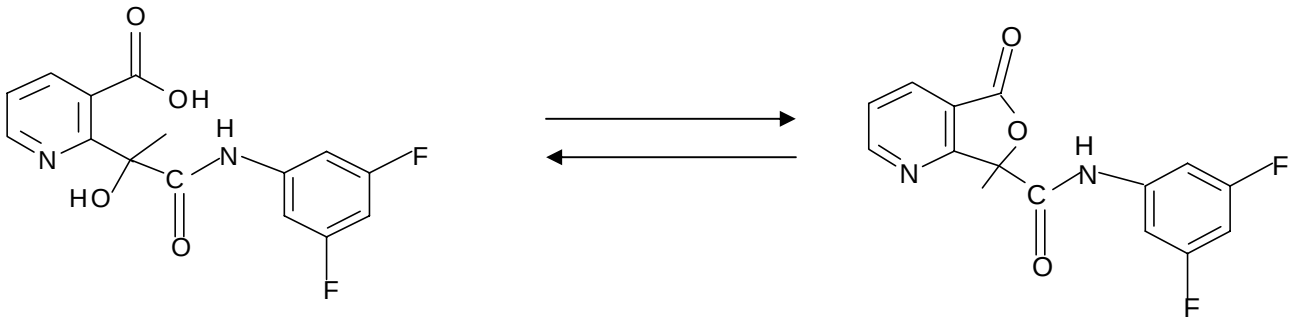
Identity of the Degradation Product P9 (~19 min HPLC Peak) – The structure of the degradation product P9 is shown in the Table IV. P9 is an artifact that results from the reaction of trihydroxymethylaminomethane (Tris buffer) and BAS 654 H. Trihydroxymethylaminomethane is present in Tris buffer solutions. The reaction between BAS 654 H and trihydroxymethylaminomethane is possible only at higher pH (≥ 7). At lower pH trihydroxymethylaminomethane will be protonated and can not act as a nucleophile. This artifact was also found in the soil photolysis study and the aerobic soil metabolism study of ^{14}C-BAS 654 H.[1, 2] Because dosing solution for these studies was prepared in Tris buffer (pH 7). The structure of this artifact in the soil photolysis study was proposed as shown below.



This structure has same molecular mass and fragment ion 122 as found in the Tris adduct. We were able to generate more of this artifact in the aqueous photolysis study and were able to get NMR spectral data which suggested absence of protons from pyridine ring. Also, we attempted a reaction in pH 9 aqueous medium using radioactive M6 and M2, but the 19 min peak was not observed. These results do not support the structure shown above.

Identity of the Degradation Product P11 (~21.5 min HPLC Peak) - The proposed structure of P11 is shown in the Scheme 2.

Scheme 2



P11 M23

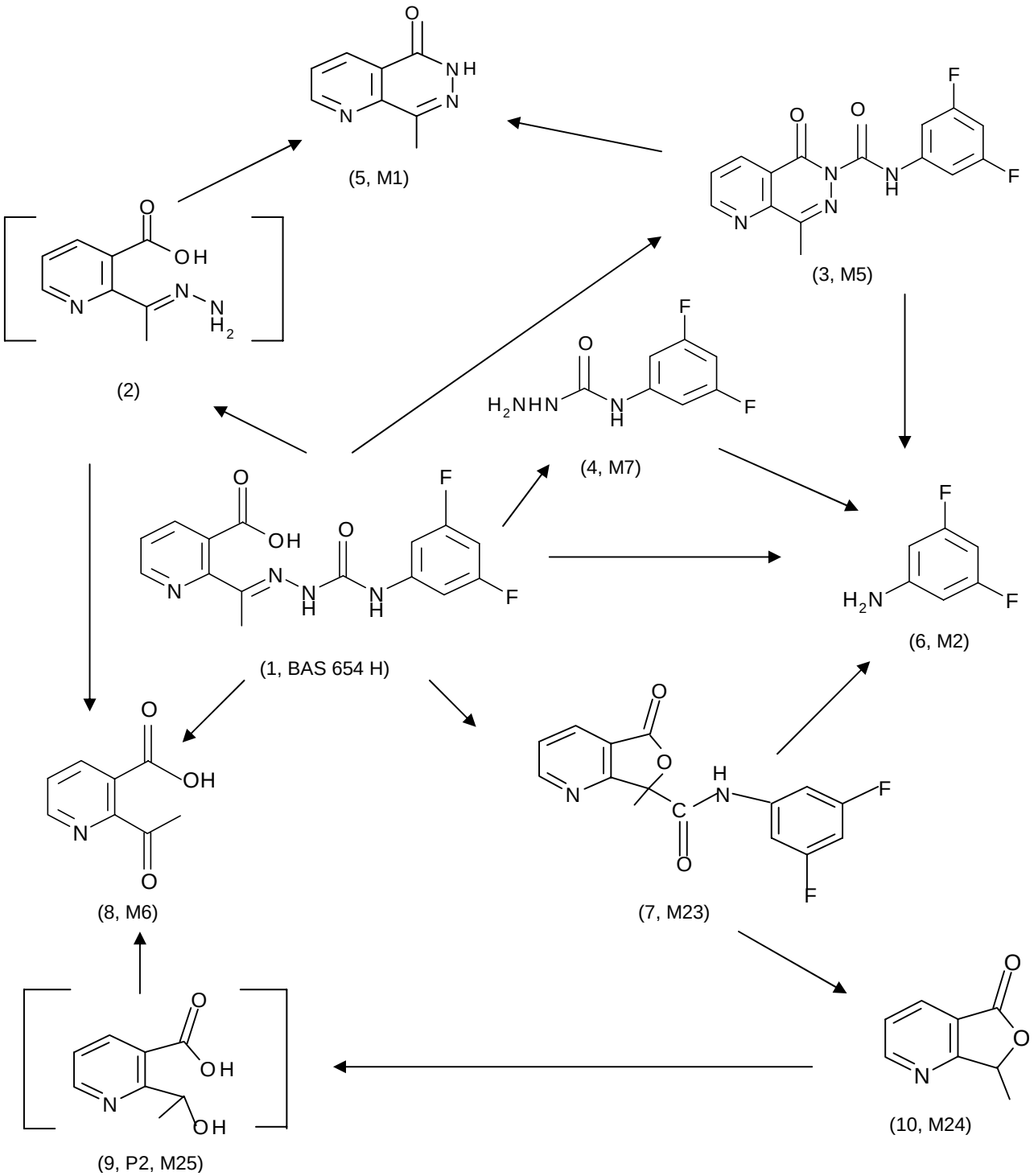
P11 is the hydrolysis product of M23. The structure assignment for P11 is based on its chromatographic and MS spectral data. The hydrolysis product P11 and M23 exist in equilibrium at pH close to neutrality. The ratio of P11 and M23 will depend on the pH of the solution. Towards lower pH, M23 will be the major product. Towards higher, pH P11 will be the major product. It is possible to drive this equilibrium to either P11 or M23. This was confirmed by dissolving ^{14}C-M23 into a catalytic amount of 0.1N NaOH which showed only P11 peak in HPLC chromatogram. When this sample was acidified to pH ~3 and reanalyzed by HPLC, it showed up as M23 peak.

Next, an aliquot of the positively identified ^{14}C-M23 (~25 min peak, isolated from pyridine label, pH 5 experiment) was dissolved into pH 9 buffer. The solution was stored at 27 °C over night and analyzed by HPLC after the storage. Two HPLC peaks corresponding to products P11 and M23 were observed in the chromatogram. The HPLC peak corresponding to retention time ~21

min was isolated and analyzed by HPLC. The HPLC chromatogram again showed 2 peaks corresponding to the retention times of P11 and M23. The isolated P11 changed into a mixture of P11 and M23 during the isolation process because the HPLC fractions which contained P11 were acidic. This sample was analyzed by LC/MS. More polar peak corresponding to P11 retention had the mass 18 units higher than M23. LC/MS of the other peak (less polar) suggested it to be M23.

Degradation Pathway – The proposed photogegradation pathway for BAS 654 H is shown in Scheme 3.

Scheme 3



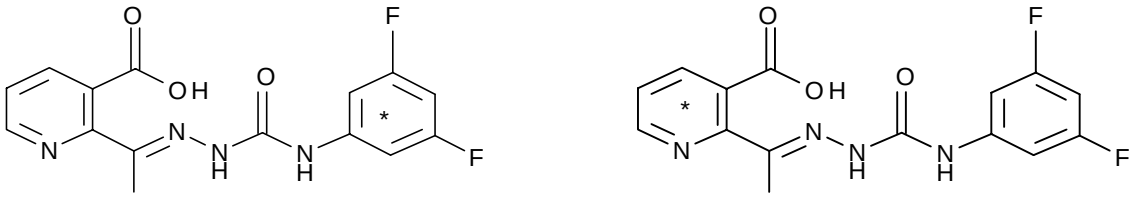
EXPERIMENTAL

A. Test Substance

The radiolabeled BAS 654 H used for the photolysis study is identified in Table V.

Table V. Characterization of BAS 654 H Used in Photolysis Study.

Common Name	Diflufenzopyr
Product Code	BAS 654 H
Specific Activity	^{14}C-BAS 654 H, Phenyl label = 255137 dpm/ug ^{14}C-BAS 654 H, Pyridinyl label = 243782 dpm/ug
Radiochemical Purity (HPLC)	^{14}C-BAS 654 H, Phenyl label = 97% ^{14}C-BAS 654 H, Pyridinyl label = 98%



The radiolabel positions in the molecule are indicated by star symbols. The phenyl ring is uniformally labelled. In the case of pyridine labelled test substance the radiolabel at positions C-4 and C-6 is evenly distributed (50:50) i.e. there is one ^{14}C-label per molecule of BAS 654 H. The radiopurity of the test substances was determined by reversed-phase high performance liquid chromatography (HPLC) before adding into the buffer solutions. The radiopurity was found to be 97% (pyridine label) and 96% (phenyl label).

B. Equipment and Setup

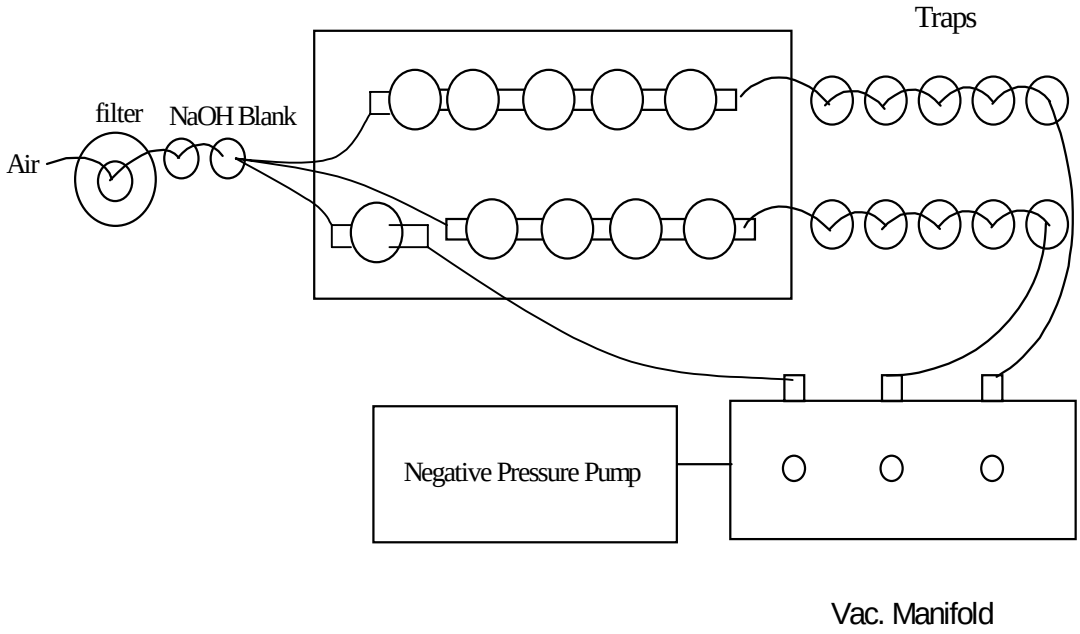
Photolysis Apparatus and Testing Procedure

Photolysis was carried out in an Atlas Suntest CPS Plus unit with a xenon lamp equipped with filters to mimic sunlight (wavelengths < 290 nm were filtered out). Continuous illumination was employed throughout the study. The light intensity between 300 – 800 nm was measured using a LI-COR model LI-1800 Spectroradiometer at the beginning and end of study for both labels. For the pyridine label study, the average light intensity (pre- and post- study) was 578 W/m^2. For the phenyl label study, the average light intensity (pre- and post- study) was 643 W/m^2. Natural sunlight intensity in the spring at 40 N latitude is 583 W/m^2 .[3]

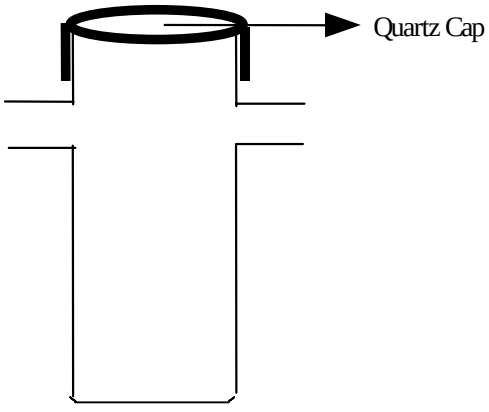
The photolysis setup consisted of a rectangular metallic hollow block equipped with coolant inlet and outlet. The thermostated block was provided with 10 wells, five in each row, for placing 10

glass photolysis vessels. The volume of each glass vessel was ~20 mL and was equipped with an air inlet and outlet. Each photolysis vessel was provided with a glass cap fitted with a quartz glass disc at the top. A vessel was filled with ethylene glycol, placed into a well and was used to measure the temperature during photo-period at the time of sampling. Four vessels were filled with ^{14}C-BAS 654 H (pyridine label) treated pH 7 buffer solution and were placed in one row. Five vessels were filled with ^{14}C-BAS 654 H (pyridine label) treated pH 9 buffer solution and were placed in the other row. Except the ethylene glycol sample, all the samples in the same row were connected to a set of trapping solutions for the collection of volatile radioactivity. The treated samples in each row had a separate set of trapping solutions. A partial picture of the photolysis setup is shown in the Figure 1.

Figure 1



Photolysis Vessel for Holding Samples



A preliminary study was conducted prior to photolysis to determine the cooling bath temperature setting required to maintain the treated solutions at 22 ± 1° C during photo-period. This was achieved by placing a photolysis glass vessel containing ~18 mL buffer solution into a well on the thermostated block with a thermocouple probe inserted into the solution. Air was allowed to flow over the buffer solution and the cooled fluid was circulated through the thermostated block. Next, the photolysis lamp was turned on and the buffer solution was irradiated continuously for >72 h. During this irradiation period the cooling bath temperature was kept at 20.9° C to maintain the buffer solution at 22 ± 1° C.

During the irradiation period a stream of sterile, CO_2 free and moistened air was purged through the vessels under negative pressure. Air exiting the reaction vessels was successively passed through a 1N NaOH trap (10 mL, for $^{14}CO_2$), a second 1N NaOH trap (10 mL, for $^{14}CO_2$) and a ethylene glycol trap (10 mL, for organic volatile. At each sampling interval, except time zero, two vessels, one from each of the treated test systems (pH 7 and pH 9), were removed for LSC and HPLC analysis. The remaining sample vessels were reconnected immediately after sample removals for continued irradiation. Volatile trapping solutions were removed at every sampling interval and were analyzed by LSC to estimate the amount of volatile radioactivity.

The process described above was also performed with ^{14}C-BAS 654 H (pyridine label) treated pH 5 buffer solution and ^{14}C-BAS 654 H (phenyl label) treated pH 5, pH 7 and pH 9 buffer solutions.

C. Test System Preparation

Procedure to Ensure Sterile Conditions and the Desired pH

All glassware and buffer solutions used were sterilized by autoclaving at 120 °C for 20 min. Test buffer solutions were filtered through 0.2 mm pore size sterile filter before autoclaving. The addition of ^{14}C-BAS 654 H to the test buffers was carried out in a laminar flow hood using aseptic conditions. Aliquots of the treated buffers were placed in photolysis vessels. The photolysis vessels containing the treated buffers were capped with quartz glass caps and were connected in series using tygon tubing. One end of the sample series was connected with a 1N NaOH solution trap and a 0.22 μm disc filter. The other end of the sample series was connected with the trapping solutions (NaOH followed by ethylene glycol). This kind of setup provided minimal contact with non-sterile environment. The sample assembly was disconnected for a brief period only at the time of time-course sample removal for analysis and was reconnected immediately after the removal of the time-course samples. To check for microbial contamination, a small amount of the treated buffer was placed on an agar plate and incubated at ambient temperature at the beginning of the experiment and after the last sampling. No microbial growth was observed on any plate indicating that the treated solutions remained sterile during the irradiation period. The pH was measured just after the treatments and at the time of analysis of the samples.

Preparation of pH 5 buffer solution

Sodium acetate (0.3284 g) was dissolved into HPLC grade water (400 mL). Next, 50 μL of acetic acid was added to the sodium acetate solution to obtain a 0.01M pH 5 buffer solution. The pH of the buffer solution was measured using a pH meter and was 5.01. The buffer pH was also measured by pH indicator strip and was 5.0. The buffer solution was filtered through a nalgene sterilized filtration unit (pore size 0.2 μm) and autoclaved at 120° C for 20 min prior to the treatment.

Preparation of pH 7 buffer solution

Trizma pre-set crystals (0.7741 g, Sigma, lot no. 64H5751) was dissolved into HPLC grade water (500 mL) to obtain a 0.01M pH 7 buffer solution. The pH of the buffer solution was measured using a pH meter and was 6.8. The buffer pH was also measured by pH indicator strip and was 7.0. The buffer solution was filtered through a nalgene sterilized filtration unit (pore size 0.2 μm) and autoclaved at 120° C for 20 min prior to dosing.

Preparation of pH 9 buffer solution

Trizma pre-set crystals (0.6230 g, Sigma, lot no. 90H5600) was dissolved into HPLC grade water (500 mL) to obtain a 0.01M pH 9 buffer solution. The pH of the buffer solution was measured using a pH meter and was 8.83. The buffer pH was also measured by pH indicator strip and was 9.0. The buffer solution was filtered through a nalgene sterilized filtration unit (pore size 0.2 μm) and autoclaved at 120° C for 20 min prior to the treatment.

D. Preparation of ^{14}C - BAS 654 H Stock Solution (pyridine label)

The stock solution was prepared by dissolving pyridine label ^{14}C-BAS 654 H (~5 mg, lot no. 980921, inventory no. 279) into a mixture of DMSO and acetonitrile (10 mL, 0.5:9.5). A 50 μL aliquot of the stock solution was diluted to 5.0 mL using acetonitrile and assayed by LSC (3 x 50 μL) to determine the exact concentration of the stock solution. A 50 μL aliquot of the dilution was analyzed by HPLC to determine the radiopurity of the test substance in the stock solution. The whole HPLC run was collected, measured and assayed by LSC (3 x 1.0 mL) to determine the recovery of the HPLC run. The radiopurity was 96.56% and the recovery of the HPLC run was 101.3%.

E. Preparation of ^{14}C - BAS 654 H Stock Solution (phenyl label)

The stock solution was prepared by dissolving phenyl label ^{14}C-BAS 654 H (~5 mg, lot no. 980921, inventory no. 278) into a mixture of DMSO and acetonitrile (10 mL, 0.5:9.5).

A 50 μL aliquot of the stock solution was diluted to 5.0 mL using acetonitrile and assayed by LSC (3 x 50 μL) to determine the exact concentration of the stock solution. A 50 μL aliquot of the dilution was analyzed by HPLC to determine the radiopurity of the test substance in the stock solution. The whole HPLC run was collected, measured and assayed by LSC (3 x 1.0 mL) to determine the recovery of the HPLC run. The radiopurity was 96.25% and the recovery of the HPLC run was 105.5%.

F. Treatment of Test Systems with ^{14}C - BAS 654 H (pyridine label and phenyl label)

For the Determination of Degradation Profile and Kinetics

A 100.0 mL aliquot of the sterile test buffer solution (pH 7) was transferred into a 250 mL Erlenmeyer flask. A 800 μL aliquot of the pyridine label stock solution was added to the test buffer solution. The treated buffer solution was thoroughly mixed by shaking for about 2 min. Then, aliquots (17±1 mL each) of the treated buffer solution were transferred into pre-weighed photolysis vessels. The photolysis vessels containing the treated buffer solutions were weighed again before and after photo-periods to obtain the exact weights of the treated samples before and after irradiation for the calculation of %TAR in the samples. Next, the samples were placed on the thermostated block. Then, samples were connected with volatile collection traps and the lamp was turned on. The samples were irradiated continuously.

Aliquots of the remaining treated buffer solution were assayed by LSC (3 x 50 µL) to determine the total applied radioactivity (TAR) in the buffer solution and this was 100% TAR. The level of the treatment for pH 7 test system was 5.36 ppm. Next, a small amount of the treated buffer was placed on an agar plate and the plate was stored at ambient temperature for the sterility check.

Approximately 1.5 mL aliquots of the treated buffer were transferred into a number of HPLC vials, tightly capped, stored under darkness in a incubator maintained at 22 ± 1° C and were used as dark controls.

The procedure for the treatment of pH 5 and 9 test buffer solutions was performed as described above. The treatment levels at pH 5 and 9 were 5.68 ppm and 5.33 ppm respectively.

The procedure for the treatment of the test systems (pH 5, pH 7 and pH 9) with phenyl labeled ^{14}C-BAS 654 H was exactly similar to that of pyridine labeled experiments as mentioned above. The treatment rates for pH 5, pH 7 and pH 9 buffer solutions were 4.90 ppm, 4.85 ppm and 4.83 ppm respectively.

For the Identification of Degradation Products

The test buffers of pH 5 and pH 9 were treated with ^{14}C - BAS 654 H (pyridine and phenyl labels) and irradiated to generate larger quantities of the degradation products to facilitate identification.

A 20 mL aliquot of the pH 5 buffer and 60 mL aliquot of pH 9 buffer were treated with 0.8 mL and 2.4 mL aliquots of the pyridine labeled BAS 654 H stock solution prepared above. The average treatment rate for both buffers was 28.12 ppm. Similarly, pH 5 and pH 9 buffers were also treated with the stock solution of phenyl labeled BAS 654 H prepared above. The average treatment rate for phenyl label was 20.42 ppm.

G. Sampling Intervals

Irradiated and dark control samples were removed at the same time for LSC and HPLC analyses. Except time zero, the trapping solutions were removed at every sampling interval for the estimation of volatile radioactivity. In the case of radioactive samples, a vessel containing the treated sample was removed at every sampling interval except zero time. The sampling intervals for all the test systems are listed below.

Pyridine Label

Sampling Intervals pH 5 (h:min) – 0, 69:55, 164:47, 259:26, 355:13, 399:31 and 689:39.
Sampling Intervals pH 7 (h:min) – 0, 116:28, 210:09, 304:33, and 475:51.
Sampling Intervals pH 9 (h:min) – 0, 116:28, 210:09, 304:33, and 475:51.

Phenyl Label

Sampling Intervals pH 5 (h:min) – 0, 116:41, 256:32, 449:07 and 570:26.
Sampling Intervals pH 7 (h:min) – 0, 116:41, 235:07, 399:26, and 641:54.
Sampling Intervals pH 9 (h:min) – 0, 116:41, 235:07, 399:26, and 641:54.

H. Quantitation and Identification of Volatile ^{14}C-Products of ^{14}C-BAS 654 H

Experimental details about the setup for the collection of volatiles is described earlier in the Equipment and Setup Section. The radioactivity in the 1N NaOH and ethylene glycol trapping solutions were determined by LSC (3 x 1 mL) assays. The volatiles trapped in 1N NaOH solutions were characterized by reacting with sulfuric acid and trapping $^{14}CO_2$ generated into Harvey cocktail. The experimental setup is described below.

An air-pump, 5N sulfuric acid trap (10 mL), reaction trap (2.0 mL aliquot from 1N NaOH trap) and a vigreux column containing Harvey cocktail (15 mL) were connected sequentially in this order. Sulfuric acid was transferred into reaction trap under positive air pressure and the liberated $^{14}CO_2$ was allowed to bubble through the Harvey cocktail. Harvey cocktail was assayed by LSC to determine the amount of $^{14}CO_2$. The reaction trap which contained the neutralized mixture of sodium hydroxide and sulfuric acid was assayed by LSC to determine the amount of other volatile radioactive products.

I. Quantitation of ^{14}C-BAS 654 H and its Degradation Products in Irradiated Test Systems

Each time-course sample was assayed by LSC (3 x 50 or 25 µL) to determine the amount of the radioactivity present in the buffer solutions. Next, a 100 µL aliquot of each time-course sample was analyzed by HPLC under the following conditions to determine the quantitative distribution of radioactivity present in the buffer solutions.

Column: YMC ODS AQ, 4.6 mm x 250 mm, 5 µm
Detector: Radiometric and UV 254 nm
Flow Rate: 1.0 mL / min
Mobile Phase: A = 0.1% TFA in Water; B = acetonitrile

Gradient:

Time (min)	%A	%B
00.0	98.0	02.0
15.0	60.0	40.0
24.0	20.0	80.0
28.0	20.0	80.0
30.0	98.0	02.0
35.0	98.0	02.0

HPLC Run Time: 35 min

J. Quantitation of ^{14}C-BAS 654 H and its Degradation Products in Dark Control Test Systems

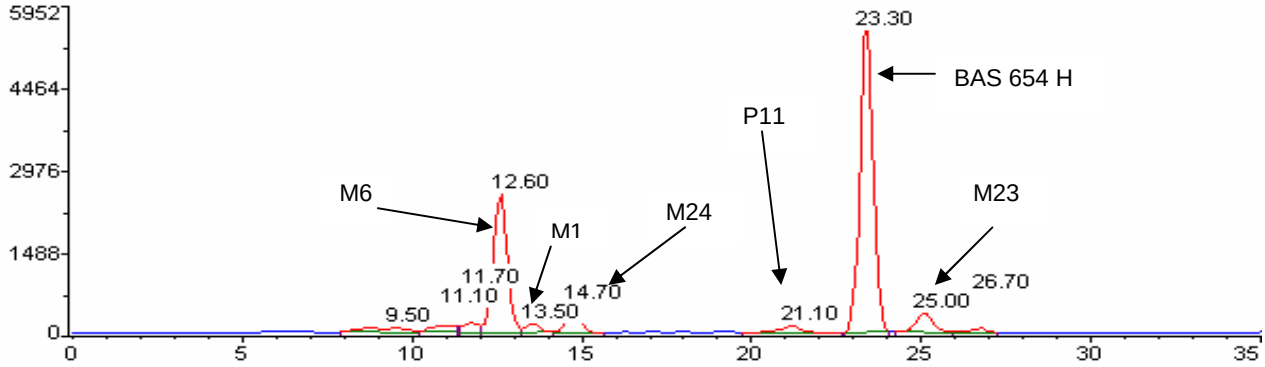
The dark control samples were analyzed concurrently with the irradiated samples. Aliquots (3 x 50 or 25 µL) of the ^{14}C-BAS 654 H (pyridine and phenyl) treated dark control samples were assayed by LSC at every sampling interval to determine the material balance. For each time-course sample a 100 µL aliquot was analyzed by HPLC to obtain the quantitative distribution profile of the hydrolysis products in the dark control samples. The HPLC analysis condition was exactly the same as listed above for the irradiated samples.

K. Isolation of ^{14}C-Products from the Treated Buffer Solution for Identification

<u>Pyridine Label</u>
Several irradiated samples (pH 5 and pH 7) were combined and concentrated to about 0.2 mL under reduced pressure on a rotary evaporator. The concentrated sample was quantitatively

transferred into a 1.0 mL volumetric flask using DMSO and acetonitrile mixture (0.6 mL, 1:2). A representative HPLC chromatogram of this sample is shown in Figure 2. Several 150 µL aliquots of this sample were injected into HPLC and 30 sec fractions were collected for the isolation of photo-degradation products.

Figure 2



Identification of HPLC Peaks ~13 min (M6) and ~14 min (M1) - The appropriate HPLC fractions, obtained from the fraction collection, were combined. The combined sample was extracted with EtOAc. An aliquot of the EtOAc extract was concentrated to near dryness under a stream of N_2 and the dried sample was redissolved into DMSO/acetonitrile (1:9). This sample was analyzed by HPLC co-chromatography with reference standards of M6 and M1. Another aliquot of the EtOAc extract was analyzed by LC/MS.

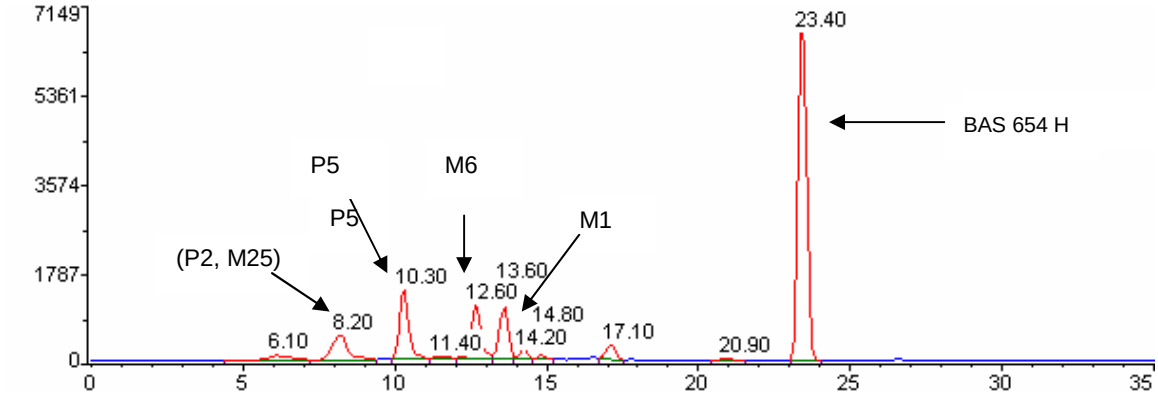
Identification of HPLC Peak ~15 min (M24) - The appropriate HPLC fractions, obtained from the fraction collection, were combined. The combined sample was extracted with EtOAc. The extract was concentrated to remove most of the EtOAc. The remaining sample was diluted with acetone. This sample was analyzed by HPLC, GC/MSD and ^{1}H-NMR.

Identification of HPLC Peak ~24 min (BAS 654 H) - The appropriate HPLC fractions, obtained from the fraction collection, were combined. The combined sample was extracted with EtOAc. An aliquot of the EtOAc extract was concentrated to near dryness under a stream of N_2 and the dried sample was redissolved into DMSO/acetonitrile (1:9). This sample was analyzed by HPLC co-chromatography with a reference standard of BAS 654 H. Another aliquot of the EtOAc extract was analyzed by LC/MS.

Identification of HPLC Peak ~25 min (M23) - The appropriate HPLC fractions, obtained from the fraction collection, were combined. The combined sample was extracted with EtOAc. An aliquot of the EtOAc extract was concentrated to near dryness under a stream of N_2 and the dried sample was redissolved into DMSO/acetonitrile. This sample was analyzed by HPLC co-chromatography with a ^{14}C-reference standard of M23 isolated and identified in soil photolysis study.[1] Another aliquot of the EtOAc extract was analyzed by LC/MS.

Several irradiated samples (pH 9) were combined and concentrated to about 0.5 mL (in vacuo under reduced pressure on a rotary evaporator). The concentrated sample was quantitatively transferred into a 5.0 mL volumetric flask using a mixture of DMSO, acetonitrile and pH 9 buffer (0.5 mL, 1.0 mL and 3.0 mL). A representative HPLC chromatogram of this sample is shown in Figure 3. Several 120 µL aliquots of this sample were injected into HPLC and 30 sec fractions were collected for the isolation of polar photo-degradation products (retention times ~8-10 min).

Figure 3



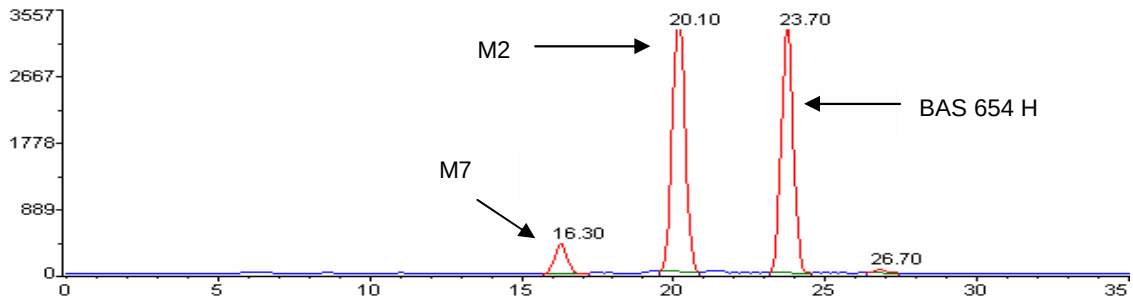
Identification of HPLC Peaks ~8-9 min Region (P2, Precursor of M24) - The HPLC fractions (10 - 14), obtained from the fraction collection, were combined, concentrated and refractionated by HPLC. The HPLC fractions 2-6 obtained from refractionation were combined, concentrated, redissolved into MeOH-d_4 and analyzed by HPLC, LC/MS and ^{1}H-NMR. The HPLC fractions 17-21 obtained from refractionation were combined and extracted with EtOAc. The EtOAc extract was concentrated to dryness, redissolved into MeOH-d_4 and analyzed by HPLC, LC/MS and ^{1}H-NMR.

Identification of HPLC Peaks ~9-10 min Region (P5, Precursors of M1) - The appropriate HPLC fractions, obtained from the fraction collection, were combined, concentrated and refractionated by HPLC. The HPLC fractions 14-17 from refractionation were combined and extracted with EtOAc. The EtOAc extract was concentrated, redissolved into acetone and analyzed by HPLC co-chromatography with a standard of M1 and LC/MS and GC/MS. The HPLC fractions 24-26 from refractionation were combined and extracted with EtOAc. The EtOAc extract was concentrated to dryness, redissolved into acetone and analyzed by HPLC, LC/MS, GC/MS and ^{1}H-NMR.

Phenyl Label

A SPE cartridge (Waters HLB, 6 mL) was conditioned by washing with MeOH (~5 mL) followed by HPLC grade water (20 mL). Then, several dark control samples (pH 5 experiment, 570 h, HPLC chromatogram, Figure 4) were passed through the preconditioned cartridge. The pre-eluate contained insignificant amount of radioactivity (LSC assay) and was discarded. Most of the radioactivity was retained on the cartridge and was eluted with acetone (5 mL). Acetone eluate was diluted with EtOAc and washed with pH 9 water. Then, approximately 100 µL of DMSO was added into the EtOAc extract and the sample was concentrated on a rotary evaporator to almost dryness. The concentrated sample was transferred quantitatively into a HPLC vial using small amount of acetonitrile. Several 75 µL injections of this sample were made into HPLC and 30 sec fractions were collected.

Figure 4

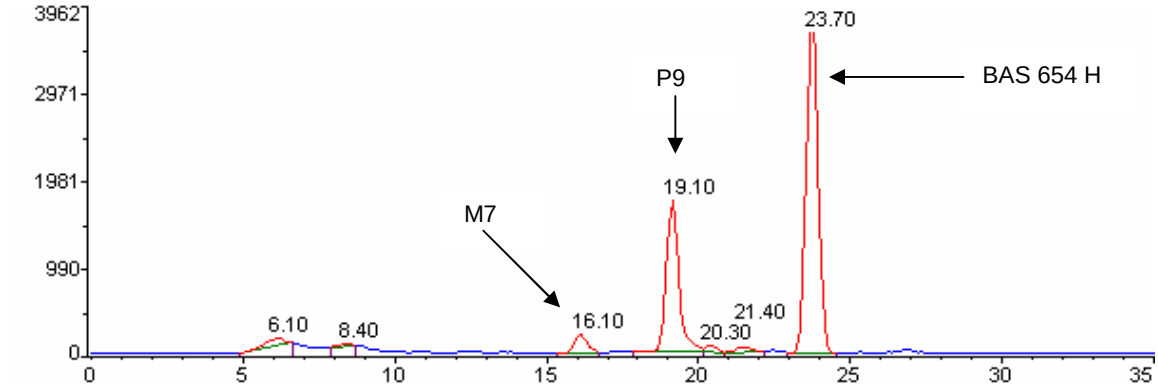


Identification of HPLC Peak ~16 min (M7) - The HPLC fractions 18-22, obtained from the fraction collection, were combined. The pH of the sample was adjusted to ~8 using 10% NH_4OH. Then, the solution was extracted with EtOAc. An aliquot of the EtOAc extract was concentrated to near dryness under a stream of N_2 and the dried sample was redissolved into DMSO/acetone (1:9, 100 µL). This sample was analyzed by HPLC co-chromatography with a reference standard of M7. Another aliquot of the EtOAc extract was analyzed by LC/MS.

Identification of HPLC Peak ~21 min (M2) - The HPLC fractions 27-29, obtained from the fraction collection, were combined. The pH of the sample was adjusted to ~8 using 10% NH_4OH. Then, the solution was extracted with EtOAc. An aliquot of the EtOAc extract was concentrated to near dryness under a stream of N_2 and the dried sample was redissolved into DMSO/acetone (1:9, 100 µL). This sample was analyzed by HPLC co-chromatography with a reference standard of M2. Another aliquot of the EtOAc extract was analyzed by GC/MSD.

A high dose irradiated sample (pH 9 experiment, ~642 h, HPLC chromatogram, Figure 5) was passed through a preconditioned SPE cartridge (Waters HLB, 6 mL). Most of the radioactivity was retained on the cartridge and was eluted with acetone (10 mL). Acetone eluate was concentrated to near dryness under a stream of N_2. The residual material was redissolved into a mixture of DMSO and acetonitrile (1:9, ~1.0 mL). Several 90-180 µL injections of this sample were made into HPLC and 15 sec fractions were collected.

Figure 5



Identification of HPLC Peak ~19 min (P9) - The HPLC fractions 30-32, obtained from the fraction collection, were combined and extracted with EtOAc. An aliquot of the EtOAc extract was concentrated to near dryness under a stream of N_2 and the dried sample was redissolved into DMSO/acetone (1:9). This sample was analyzed by HPLC. Another aliquot of the EtOAc extract was concentrated to dryness, redissolved into MeOH-d_4 and analyzed by LC/MS and ^{1}H-NMR spectroscopy.

The HPLC fractions 36-39, obtained from the fraction collection, were combined and extracted with EtOAc. An aliquot of the EtOAc extract was concentrated to near dryness under a stream of N_2 and the dried sample was redissolved into DMSO/acetone (1:9). This sample was analyzed by HPLC cochromatography with a reference standard of M4. Another aliquot of the EtOAc extract was concentrated to dryness, redissolved into acetonitrile and analyzed by LC/MS.

Half-Life - Estimation of the half-life of BAS 654 H was done employing the %TAR values for BAS 654 H at various sampling intervals. Half-life calculations were based on the assumption that

photolysis degradation and dark control degradation (hydrolysis) followed first order kinetics. The equation employed for this determination was as follows:

$$C = C_o e^{-kt}$$

where: C = concentration of BAS 654 H (%) at time t
C_o = concentration of BAS 654 H (%) at time 0
e = exponential function
k = photolysis/hydrolysis rate constant
t = time in hours

ACKNOWLEDGEMENT

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