I. SUMMARY AND INTRODUCTION

A. SCOPE

This method is to be used for the semiquantitative determination of CGA-77102 ([Acetamide, 2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(2-methoxy-1-methylethyl)-,(*S*)-], CAS # 87392-12-9) in water. The limit of detection (LOD, the smallest dose that yields a response that is statistically significant different than the response of the zero dose) is 0.05 ppb of CGA-77102. The limit of quantitation (LOQ, the lowest level of fortification with which an acceptable recovery can be obtained) for the method is 0.10 ppb of CGA-77102.

B. PRINCIPLE

A 100-µl aliquot of a representative water sample is added to a microwell coated with CGA-77102 antibody. The assay is carried out by sequential addition of enzyme conjugate, wash solution, enzyme substrate, and stop solution. The reaction is terminated by acidification. Quantification is performed spectrophotometrically at 450 nm. A flow diagram for the method is presented in Figure 1.

II. MATERIALS AND METHODS

A. APPARATUS

1.0 CGA-77102 Plate kit, enzyme immunoassay microwell assay, Beacon Analytical Systems, Scarborough, ME, catalog #CPP-005.

2.0 Microtiter plate reader, Titertek Multiskan MCC/340 MK II, ICN catalog #78-626-00, or equivalent.

3.0 AUTOmate™ microplate software and manual, ICN cat. #78-599-02 and 78-599-98, respectively, or equivalent.

4.0 Microtiter plate shaker, Lab-Line Instruments catalog #4625 or equivalent.

5.0 ELP-40 Microplate Strip Washer, Bio-Tek Instruments, Bio-Tek Part No. 4091000, or equivalent (use of equipment is optional).

6.0 Eight-channel pipette capable of dispensing 100-µl volumes, Elkay Labsystems catalog #4142-417 or equivalent.

7.0 Adjustable microliter pipette, capable of dispensing 20- to 200-µl volumes, Rainin cat. #P-200 or equivalent.

8.0 Pipette tips for eight-channel pipette for volumes of 20- to 200-µl, packaged in an eight by twelve array, Costar cat. #4865 or equivalent.

9.0 Pipette tips for single-channel pipette for volumes of 100- to 1000-µl, packaged as 100 tips per rack, Costar cat. #4867 or equivalent.

10.0 Graduated pipette, 5- and 10-mL, Fisher cat. # 13-660E and 13-660F or equivalent.

11.0 Microtiter assay plate, 96 well, flat bottom, polystyrene, non-sterile, not treated for cell culture work, Flow Laboratories cat. #76-102-05 or equivalent.

12.0 Reagent reservoirs for multichannel pipettes, non-sterile, ICN Cat. #77-824-01 or equivalent.

13.0 pH meter, Corning M-90 pH Stick, VWR cat. #34102-081 or equivalent.

14.0 Magnetic stir bar, 0.5-1.0 inch in length, Fisher cat. #14-511-61 or 14-511-63 or equivalent.

15.0 Magnetic stirrer, Fisher cat. #11-496-21 or equivalent.

16.0 4-oz Amber Boston round bottles, Fisher cat. #03-320-4B or equivalent.

B. REAGENTS

1.0 Distilled, deionized water (H_2O).

2.0 Acetonitrile (ACN), HPLC Grade, Fisher cat. #A998SK-4 or equivalent.

3.0 Sodium Chloride (NaCl) crystal, certified A.C.S., CAS No. 7647-14-5, Fisher cat. # S271-500 or equivalent.

4.0 Polyoxyethylene-sorbitan monolaurate (TWEEN 20). Sigma cat. #P-1379 or equivalent.

5.0 50% H_2O/TWEEN 20 – Combined 1 mL of TWEEN 20 and 1 mL of H_2O.

6.0 H_2O/TWEEN 20 wash solution – Dissolve 9 g of NaCl and 1 mL of 50% H_2O/TWEEN 20 in 1 liter of H_2O.

7.0 Enzyme conjugate, included in the CGA-77102 plate kit, Beacon Analytical Systems.

8.0 Substrate solution, included in the CGA-77102 plate kit, Beacon Analytical Systems. Alternatively, a commercially available horseradish peroxidase tetramethylene benzidine substrate, such as "K-Blue" (Neogen Corporation cat. #300177) may be used.

9.0 Stop solution, included in the CGA-77102 plate kit, Beacon Analytical Systems. Alternatively, 1.0 N HCl, prepared by the analyst, may be used.

10.0 CGA-77102 analytical standard, Novartis Crop Protection, P.O. Box 18300, Greensboro, NC 27419-8300. Storage conditions: frozen.

C. ANALYTICAL PROCEDURE

The antibody-coated microwells, all reagents and sample and standard solutions must be warmed to room temperature, approximately 22°C, prior to use. The reaction kinetics are temperature dependent. Be certain all solutions are warmed to room temperature before running the assay.

1.0 Sample Preparation

1.1 Preparation of Water Samples

1.1.1 Water samples are received and stored refrigerated until use.

1.1.2 The pH of each sample should be measured. Set the sample container on a magnetic stirrer and add a small stir bar. Allow the sample to stir for a few seconds prior to inserting the electrode. Record the value obtained. Samples having a pH value between 5.0 and 9.0 may be analyzed by this method. Samples having

a pH outside this range are unsuitable for this assay.

2.0 Enzyme Immunoassay

2.1 Inhibition of Enzyme Conjugate

2.1.1 Approximately 200 µl of each sample or standard solution is added individually to wells of the uncoated reservoir plate as indicated on the plate layout chart previously completed by the analyst. A sample chart is illustrated in Figure 2.

2.1.2 Decant the enzyme conjugate solution into a multichannel pipette reagent reservoir.

2.1.3 Adjust an eight-channel pipette to deliver 100 µl (refer to the pipette operation manual or applicable Novartis Crop Protection SOP for operation of the multichannel pipette). Snugly fit eight tips to the pipette and transfer a 100-µl aliquot from column 1, rows A through H, of the reservoir plate to the corresponding column on the antibody-coated assay plate. Eject the used tips to a waste receptacle. Attach eight new tips and proceed to transfer aliquots from columns 2 through 12 of the reservoir plate to the assay plate taking care to change tips after each transfer.

If an alternative, equivalent pipetting device is used in lieu of a hand-held multi-channel pipette, the 100-µl aliquot from each well of the reservoir plate is transferred to the assay plate in a manner consistent with the operation of the device.

2.1.4 As soon as all sample and standard solutions have been transferred to the assay plate, successively pipette 100-µl aliquots of the enzyme conjugate solution from the reagent reservoir to each column of the assay plate from left to right as

described above. The pipette tips must be changed after each transfer.

Note: The enzyme conjugate solution has a surfactant-like character and tends to bubble and foam when pipetted. Through preliminary trials the analyst should ensure that manual or automated pipetting devices reproducibly deliver 100-µl aliquots without bubbling or foaming.

2.1.5 Place the plate on the plate shaker. Start the shaker with the power control set to the "constant" position. The speed setting should be adjusted to approximately ninety oscillations per minute or slightly less than "2" on the Lab Line shaker. Allow the plate to shake for one hour.

Note: The reaction kinetics of this assay are temperature dependent. To avoid exposing the plate to harsh fluctuations in temperature such as strong drafts, the plate shaker should be placed inside an opaque closed chamber. Placing a cardboard box over the shaker will suffice if a more elaborate apparatus, such as a darkened Plexiglas chamber, is not available.

2.2 Wash

2.2.1 Remove the plate from the shaker. Using a hand-held multichannel pipette, remove the reactants from each column left to right across the plate.

2.2.2 Starting with column one, add approximately 200 µl of wash solution to the each well, gently agitate the plate and immediately remove the wash solution. Proceed across the plate from column two to twelve washing each column in a similar fashion. Wash the plate two additional times.

2.2.3 After the final wash, shake the plate vigorously to remove most of the remaining liquid. The plate may be inverted and blotted with dry paper towels. Blot only the exterior of the plate. Do not insert the blotting paper into the wells. Visually examine the plate to ensure little wash remains.

Should only part of the microtiter plate be used, the analyst should exercise care not to contaminate unused wells.

If an alternative washing device, such as the ELP-40 microplate strip washer, is used, be certain the reactants are removed in a manner consistent with their addition.

2.3 Color Development

2.3.1 Add 100 µl of substrate to each well across the plate in the same fashion as the previous solutions were dispensed. Tips need not be changed between additions.

2.3.2 Place the plate on the plate shaker and shake for 0.5 hour with the controls set at the same settings as described in Section II.C.2.1.5. Individual wells will gradually turn varying shades of blue.

2.3.3 Terminate color development by adding 100 µl of stop solution to each well in the same fashion as the color reagent was added. Mix the acidified solution well by repeated pipetting and dispensing of each well's contents, taking care not to form bubbles or to leave liquid behind in the pipette tips. Should bubbles form, they can be broken by manipulation with a pipette tip. Tapping the side of the strip holder gently may aid mixing of an acidified solution. A well-mixed solution will appear yellow to the eye with no traces of blue remaining. Be sure to change tips

between each addition to avoid contaminating a column of wells with residue from a previous column.

The absorbance of the final reaction product within each microwell should be analyzed within 30 minutes of addition of stop solution.

D. INSTRUMENTATION

1.0 Description and Operating Conditions

1.1 The Titertek Multiskan MCC/340 MK II eight-channel filter spectrophotometer is used to measure the absorbance of the final reaction solutions in each well. The operator should select filter 4 (450 nm) for this method. The instrument should be warmed up for ten minutes prior to use. Refer to the instrument operating instructions for further details on the operation of this instrument.

2.0 Standardization

2.1 Each assay consists of standards and samples run concurrently on the same plate. The absorbance values obtained from sample solutions may only be compared to the absorbance of standards run in the same analytical set. CGA-77102 standards range from 4.0 to 0.05 ng/mL in addition to a H_2O blank (zero dose).

2.2 Spectrophotometric analysis of the colored reaction products obtained from standard solutions will yield absorbance values measured at 450 nm (A_{450}). With a calculator or computer, use these data to generate a log/linear regression function. This curve is in the form of $y = m \log(x) + b$. The concentrations of the CGA-77102 standards are plotted on a logarithmic scale on the horizontal (x) axis and the absorbance values are plotted on a linear scale on the vertical (y) axis. An example of a typical standard curve is shown in Figure 3.

E. INTERFERENCES

1.0 The antibodies used in this assay bind primarily to CGA-77102 and metolachlor. Refer to Section III.A.2.0.

SPECIFICITY for a detailed discussion of the cross-reactivity parameters of this assay.

2.0 Slight inhibition was observed when glassware was not rinsed before use. Therefore, to obtain best results, all glassware must be pre-rinsed before use. This includes volumetric flasks, bottles, and scintillation vials used in the preparation of standards, beakers used to contain distilled water, and any other glassware that may be used. Pre-rinsing involves triple rinsing with Acetone and blowing dry with Nitrogen air. Vessels used to contain water should then be rinsed three times with de-ionized water before filling with distilled water.

F. CONFIRMATORY TECHNIQUES

1.0 The ability of the microwell assay to respond to CGA-77102 in water may be assessed by the analysis of samples fortified with a known amount of CGA-77102.

G. TIME REQUIRED

An analyst can analyze forty-two samples and six standards in duplicate in approximately three hours.

H. MODIFICATIONS AND POTENTIAL PROBLEMS

1.0 As previously stated in the introduction to the ANALYTICAL PROCEDURE, the microwell assay plates, reagents and sample solutions must be warmed to room temperature, approximately 22°C, before use. Placing the plate (in its plastic bag) and the reagents in a hood with the sash drawn low for approximately 30 minutes will bring the reagents to temperature efficiently.

2.0 The pH of the water sample, if extreme, may affect antibody binding. The pH of each sample should be examined. If a sample is found to have a pH outside the range of 5.0 - 9.0, it is unsuitable for analysis by this method.

3.0 The bottom surface of the microwell strips is the optical surface through which the absorbance of the final reaction product will be measured. The analyst should exercise care to prevent damage to this surface. The strip holder is designed to prevent the bottom of the strips from contacting flat surfaces, such as a bench top, upon which the strip

holder may be placed. Nevertheless, the analyst should maintain a clean work area as a preventative measure.

4.0 The analyst should take care to be certain all sample and standard solutions are positioned properly in the reservoir plate. The large sample load requires that special attention to detail be maintained throughout the analysis.

5.0 The analyst may observe absorbance values which are less than those of the highest standard, 4.0 ng/mL. In this event, the concentration of the corresponding sample cannot be calculated since its absorbance readings do not fall within the range of the standard curve. The sample should be diluted and re-assayed to obtain absorbance readings which lie within the bounds of the standard curve. The concentration of the undiluted sample can be calculated by multiplying the concentration of the diluted sample by the dilution factor.

6.0 The transfer of solutions by multichannel pipettes requires the analyst to constantly monitor his or her technique. Pipetting errors are the major source of error in immunoassay methodology.

I. PREPARATION OF STANDARD SOLUTIONS

Dissolve 5.0 mg of CGA-77102 in a minimal amount of ACN (approximately 15 mL), sonicate, and bring volume up to 100 mL with H_2O. This will make 100 mL of a 50 µg/mL solution. Serially dilute this solution with H_2O to make 5000-, 1000-, 10-, 4.0-, 1.0-, 0.3-, 0.1-,and 0.05-ng/mL standards. Prepare a blank consisting solely of H_2O. All standard solutions should be stored in amber Boston round bottles. Store these solutions at 4°C when not in use.

J. METHODS OF CALCULATION

CGA-77102 residues in sample solutions are determined by inserting the absorbance value of a given sample into the log/linear regression function generated by methods described in Section II.D.2.2. These calculations may be made on a computer or hand-held calculator.

B. LIMITATIONS

1.0 This method is intended to complement, not replace, chromatographic analyses. It can be used as an inexpensive screening technique to remove samples that yield responses below a pre-selected level of concern from further, more expensive analytical procedures.

2.0 As stated in Section II.H.2.0, the pH of a sample may affect antibody binding. Consequently, the pH of all samples must be checked prior to analysis. If the pH of a sample is outside the range of 5.0 – 9.0, that sample is unsuitable for analysis by Method 1004-98.

3.0 The cross reactivity data presented in Table 2 is not meant to be an exhaustive evaluation of the antibodies used in Method 1004-98. The twenty-one test substances screened represent a limited attempt to investigate the cross reactivity of the assay. Therefore, analysts should confirm residues of concern determined by this method by an additional analytical method.

C. CIRCUMSTANCES AFFECTING THE STUDY

There were no adverse circumstances affecting the quality or integrity of the data.

FIGURE 1. FLOW DIAGRAM FOR METHOD 1004-98

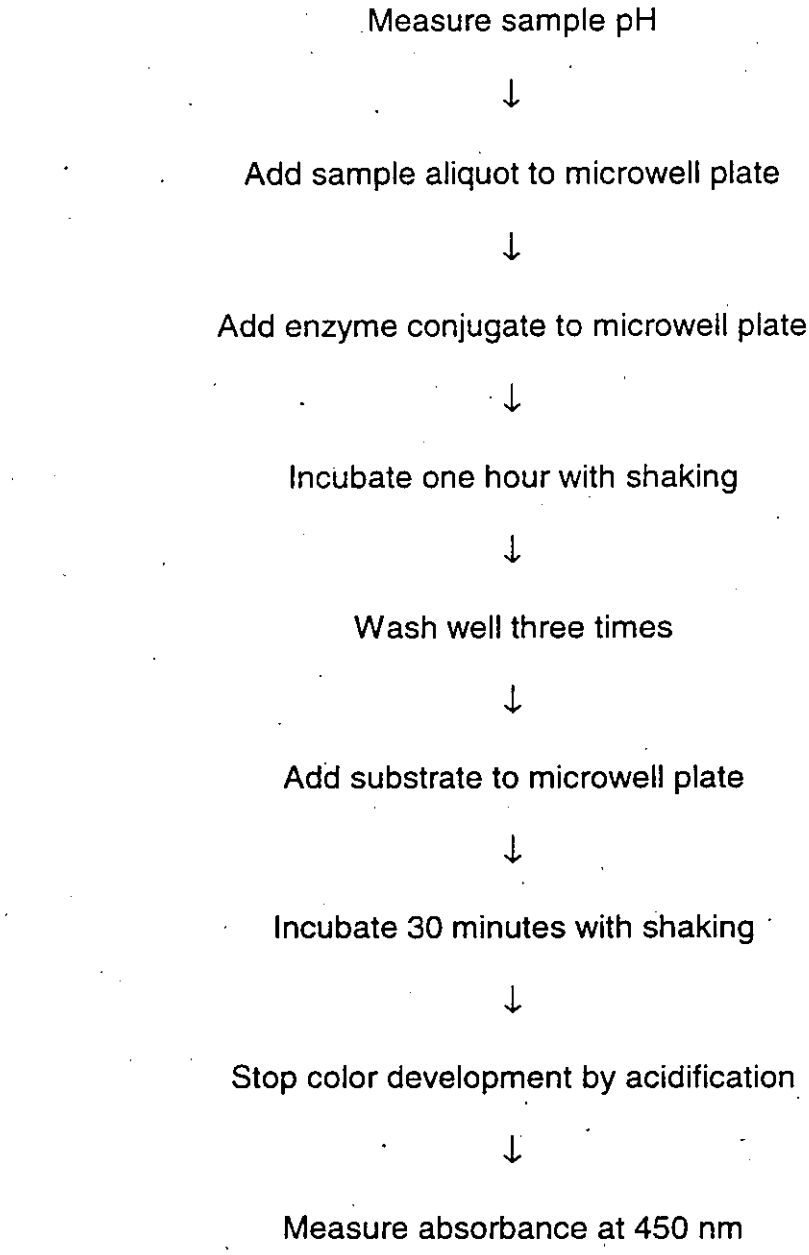
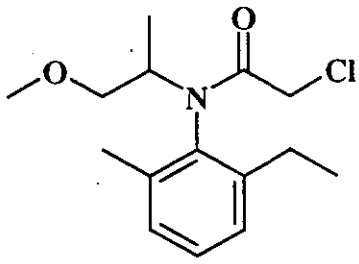
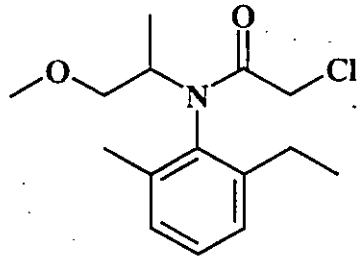


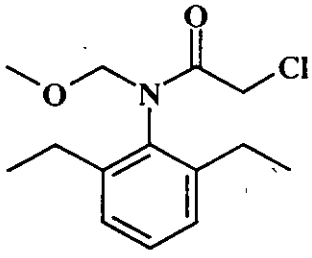
FIGURE 4. STRUCTURES OF TEST SUBSTANCES EVALUATED FOR CROSS-REACTIVITY



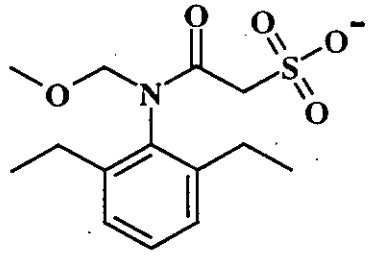
CGA-77102
(*S*-CGA-24705)



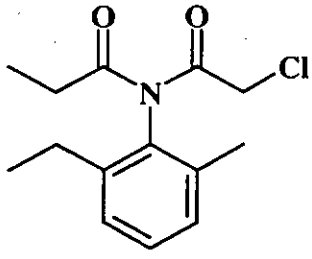
Metolachlor
(CGA-24705)



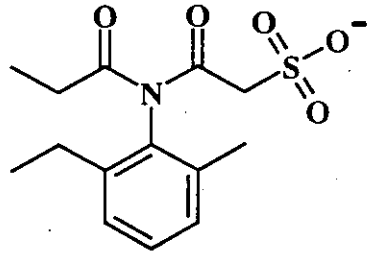
Alachlor



Alachlor-ESA

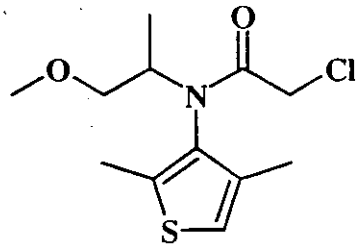


Acetochlor

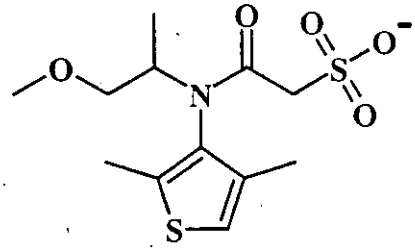


Acetochlor-ESA

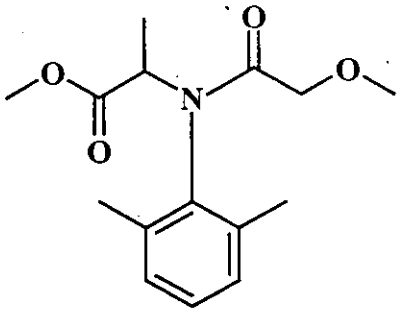
FIGURE 4. STRUCTURES OF TEST SUBSTANCES EVALUATED FOR CROSS-REACTIVITY (continued)



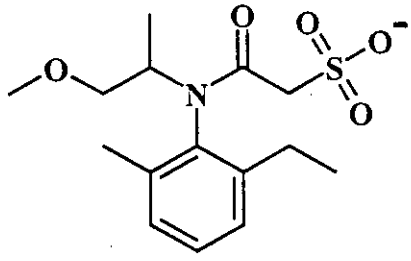
Dimethanamid



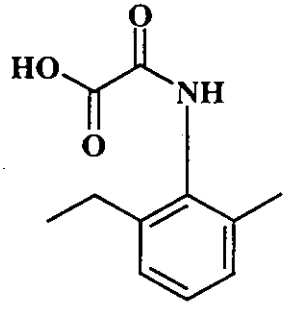
Dimethanamid-ESA



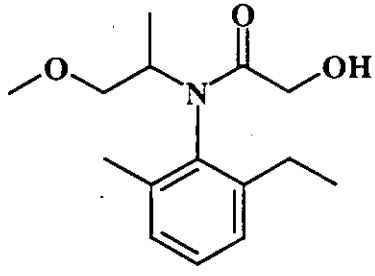
Metalaxyl



CGA-354743

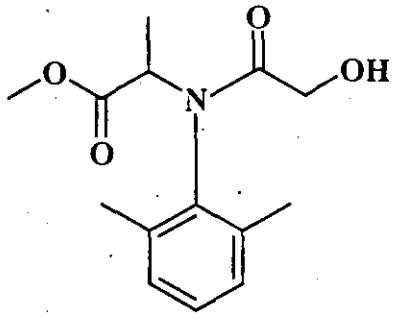


CGA-50720

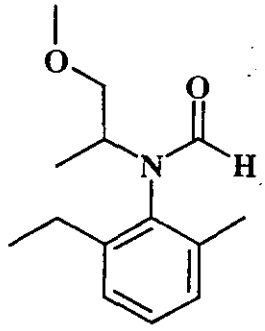


CGA-40172

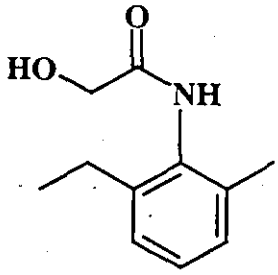
FIGURE 4. STRUCTURES OF TEST SUBSTANCES EVALUATED FOR CROSS-REACTIVITY (continued)



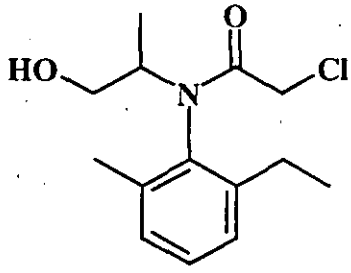
CGA-67869



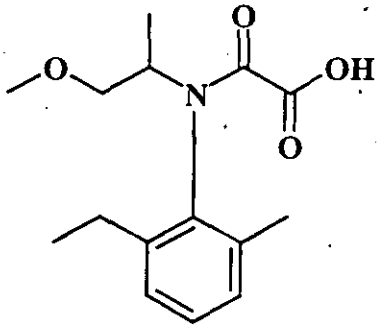
CGA-67125



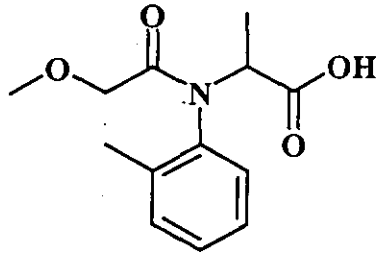
CGA-37735



CGA-41638

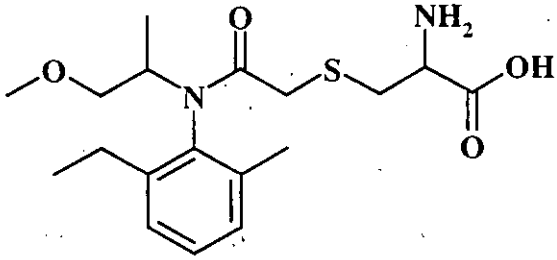


CGA-51202

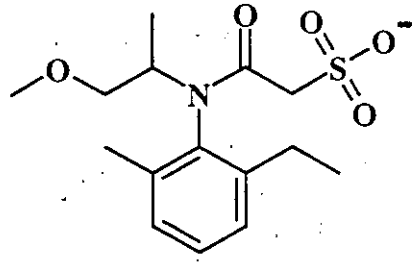


CGA-62826

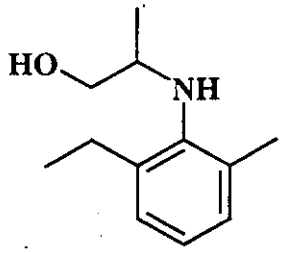
FIGURE 4. STRUCTURES OF TEST SUBSTANCES EVALUATED FOR CROSS-REACTIVITY (continued)



CGA-46576



CGA-380168
(*S*-CGA-354743)



CGA-37913