Metabolism of Tordon Herbicide (4-Amino-3,5,6-trichloropicolinic Acid) in Cotton and Decomposition in Soil

RICHARD W. MEIKLE, ELINOR A. WILLIAMS, and CARL T. REDEMANN

Bioproducts Department, The Dow Chemical Co., Walnut Creek, Calif.

Carboxyl-labeled 4-amino-3,5,6-trichloropicolinic acid-^{14}C was synthesized in 48% yield by means of a reaction between 4-amino-2-bromo-3,5,6-trichloropyridine and cuprous cyanide-^{14}C followed by alkaline hydrolysis. The radioactive compound had a specific activity of 10 mc. per mmole and was 100% radiochemically pure. 4-Amino-3,5,6-trichloropicolinic acid-^{14}C was metabolized by both cotton plants and microorganisms in soil and the rate was determined by measuring evolved radioactive carbon dioxide. In the case of the plants, the rate was very slow. Tordon herbicide was found in the cotton plants. It was also found associated with insoluble protein at a level representing only 3% of the total radioactivity; it was liberated from the protein on acid hydrolysis.

THE use and effectiveness of Tordon herbicide (Dow Chemical Co.) which contains 4-amino-3,5,6-trichloropicolinic acid have been reported (*1*). However, no information has appeared concerning its metabolism in plants and soils. This report presents the results of such a study using carboxyl-labeled 4-amino-3,5,6-trichloropicolinic acid, cotton, and a sandy loam soil.

Experimental

4 - Amino - 3,5,6 - trichloropicolinic-Carboxy-^{14}C Acid. To a warm solution (60° C.) of copper sulfate pentahydrate (1.09 mmoles per ml., 0.49 ml., 0.535 mmole) containing 0.02 ml. of very dilute sulfuric acid were added sodium metabisulfite solution (56 mg. in 0.2 ml. of water, 0.294 mmole) and then potassium cyanide-^{14}C solution (35.1 mg., 0.54 mmole, specific activity 10 mc. per mmole), followed by washings. After being warmed about 10 minutes at 60° C., the mixture was centrifuged and the solid was washed twice each with hot water, ethanol, and *n*-hexane, in that order. When the last traces of hexane had evaporated, there remained a white granular product, cuprous cyanide-^{14}C.

4 - Amino - 2 - bromo - 3,5,6 - trichloropyridine (148 mg., 0.536 mmole) and dimethylformamide (5.0 ml. distilled from cuprous chloride) were added to the cuprous cyanide-^{14}C preparation in a 50-ml. centrifuge tube. The tube was fitted with a cold-finger condenser and the contents were heated under reflux for 3 hours. At the end of this time, water (37 ml.) was added to the cooled reaction mixture and the precipitate was separated in the centrifuge and washed once with water.

The crude product from the previous reaction was heated in a sealed tube, with shaking, at 100° C. for 3 hours with 10% sodium hydroxide solution. The pH of this hydrolysis mixture was then adjusted to 2.5 with hydrochloric acid and the mixture was extracted continuously with ethyl acetate until no more radioactivity was removed. The crude solid product remaining after evaporation of the solvent was purified by column chromatography using 20 grams of Dicalite, buffered to pH 5.6 with 8 ml. of 1*M* phosphate buffer, as absorbent and ethyl acetate saturated with the same buffer as developer. The column dimensions were 0.75 × 7 inches.

The yield of carboxyl-labeled material was 61 mg., 48%; specific activity was 10 mc. per millimole. The product was 100% radiochemically pure as determined by paper chromatography in three solvent systems described below.

Greenhouse Logistics. The soil used in these experiments was a Hanford fine sandy loam with the following composition: sand, 75%, silt, 19%, clay, 9%, organic matter, 1.1%, and pH 7.0.

Small plastic pots (6.9-sq. inch surface area) filled to a depth of 2 inches with soil were planted to Alcala 4–42 cotton, and after 21 days in the greenhouse the plants were thinned to 2 per pot. The soil in each pot was then treated with 40 μg. of 4-amino-3,5,6-trichloropicolinic acid-^{14}C, 1.54 μc. of radioactivity, as a drench in 25 ml. of water containing a very small amount of ammonia.

Three experiments involving carbon-^{14}C dioxide evolution were conducted: one in which only the plants were enclosed, another in which only the pots were enclosed, and a third in which bare soil was used. In each experiment, two pots were used. The experiments were not replicated.

The pots were watered twice daily in such a manner that there was no leaching. The one exception to this regimen involved the bare soil experiment; in this case, the pots were watered only when necessary to maintain a slightly damp surface.

The experiments were carried out in the greenhouse where the temperature was maintained at 78° to 82° F.

Conversion of Barium Carbonate-^{14}C Count Rates to Microcuries per Milligram. Respired carbon dioxide was collected as barium carbonate and counted at infinite thickness using a thin end-window GM tube. The regression equation for conversion of barium carbonate-^{14}C count rates to microcuries per milligram is given by the following equation:

$$\text{Microcuries per milligram} = (5.87 \text{ c.p.m.} - 5.09) \times 10^{-7}$$

The standard deviation from regression is 34×10^{-7} and the standard deviation of the regression coefficient is 0.029×10^{-7}.

Extraction and Fractionation of Cotton Plants. Cotton plants were harvested after 14 days. The roots were washed thoroughly free of soil in running tap water and separated from the rest of the plant. All of the plant parts were blotted free of water and weighed: roots, 1.48 grams, leaves and stems, 11.95 grams.

The leaf and stem tissue was cut into small pieces, mixed with solid sodium bicarbonate, and extracted continuously for 5½ hours with *n*-hexane. The hexane extracts were dried over anhydrous magnesium sulfate and then concentrated to dryness, leaving 53 mg. of a yellow-green oily solid. The total residue was assayed for radioactivity. The radioactivity present in the hexane extract was too small to contribute significantly less than 0.01 p.p.m.

Figures 1, 2, and 3 show the subsequent disposition of the hexane-extracted leaf and stem tissue and of the roots. The values in the figures indicate the distribution of radioactivity in terms of parts per million concentration of Tordon in the total plant.

The hexane-extracted leaf and stem tissue, and the roots, were extracted separately and continuously with 80% methanol for 17 hours. The roots were first cut up into small pieces and mixed with a small amount of solid sodium bicarbonate before extraction.

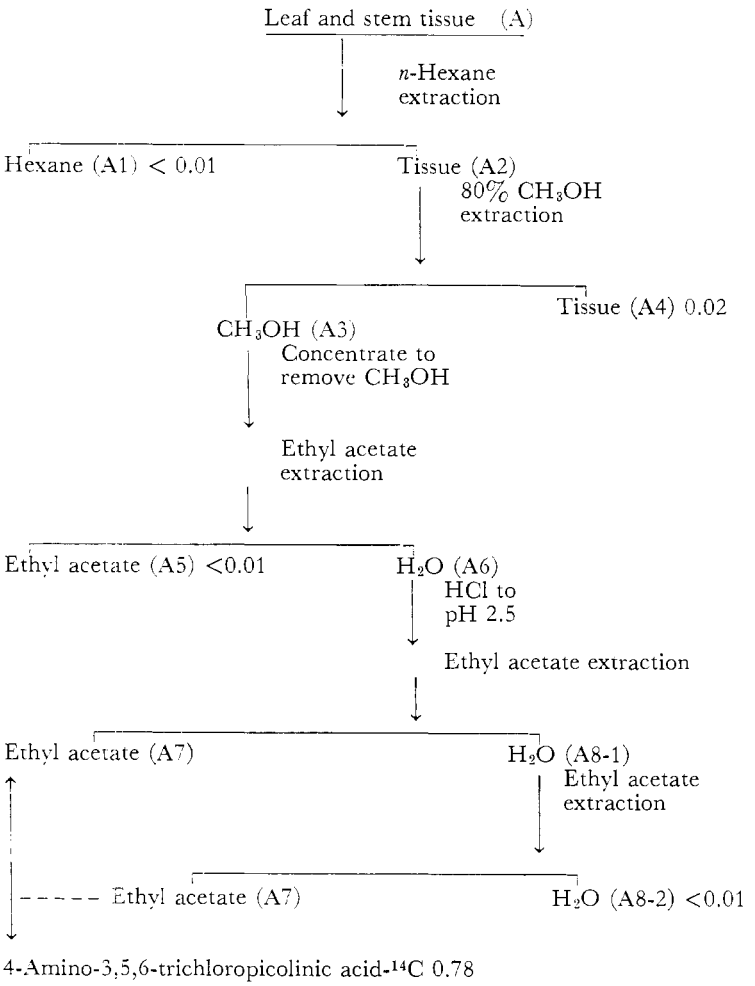

Figure 1. Fractionation procedure applied to leaf and stem tissue

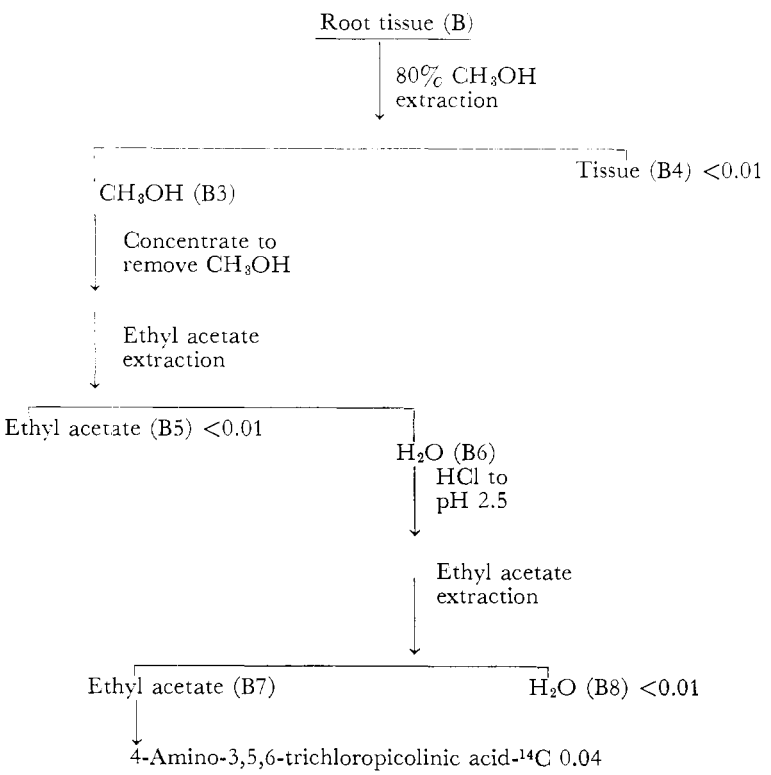

Figure 2. Fractionation procedure applied to root tissue

Both of the 80% methanol extracts were concentrated in vacuo until all the methanol was removed. The alkaline aqueous mixtures were then extracted twice with equal-volume portions of ethyl acetate and the extracts were dried over anhydrous magnesium sulfate (fractions A5 and B5).

The extracted alkaline phases (fractions A6 and B6) were adjusted to pH 2.5 with hydrochloric acid and extracted separately with 5 equal-volume portions of ethyl acetate, and the extracts were dried over anhydrous magnesium sulfate (fractions A7 and B7).

The extracted water phase of the leaf and stem tissue (fraction A8-1) was redissolved in water and extracted continuously with ethyl acetate for 7 hours. It was again evaporated to dryness in vacuo, weighed, and counted; this is now fraction A8-2. The additional ethyl acetate extraction of fraction A8-1 removed more radioactivity, and the residual material in the aqueous phase, represented by fraction A8-2, now contained less than 0.01 p.p.m. Tordon equivalent. The two ethyl acetate extracts (fraction A7 of Figure 1) were combined.

All of these ethyl acetate extracts, and also the extracted water phase (fraction B8 of Figure 2) were evaporated to dryness in vacuo and assayed for radioactivity.

The methanol-extracted leaf and stem tissue (fraction A4 of Figure 1) was again extracted continuously with 80% methanol for 17 hours as shown in Figure 3. No additional radioactivity was removed from the tissue. It was then extracted continuously with 10% acetic acid for 16 hours. The extracts were concentrated to dryness in vacuo and the residue was assayed for radioactivity. Finally, the tissue was stirred with 20 ml. of 5% trichloroacetic acid solution at reflux for 2 hours. The cooled mixture was filtered. The precipitate was washed with 5% trichloroacetic acid, water, and acetone, and the dried brown powder was assayed for radioactivity.

The trichloroacetic acid–extracted tissue (fraction A12 of Figure 3) was heated under reflux with 50 ml. of 6*N* hydrochloric acid for 17 hours. The hydrolysis mixture was cooled and filtered, and the precipitate was well washed with water followed by acetone to give a black powdery residue (fraction A14). This was assayed for radioactivity.

The acid hydrolyzate (fraction A13) was evaporated to dryness in vacuo, using several infusions of water followed by evaporation to remove residual hydrochloric acid. The residue was then dissolved in water, the pH was adjusted to 3.0 with sodium hydroxide solution, and the aqueous mixture was extracted continuously with ethyl acetate for 8 hours. The ethyl acetate extract was concentrated to a small volume in vacuo (fraction A15 of Figure 3), and the extracted aqueous phase was likewise evaporated to dryness (fraction A16). These were assayed for radioactivity.

Identification of Metabolites from Cotton. The 4-amino-3,5,6-trichloro-

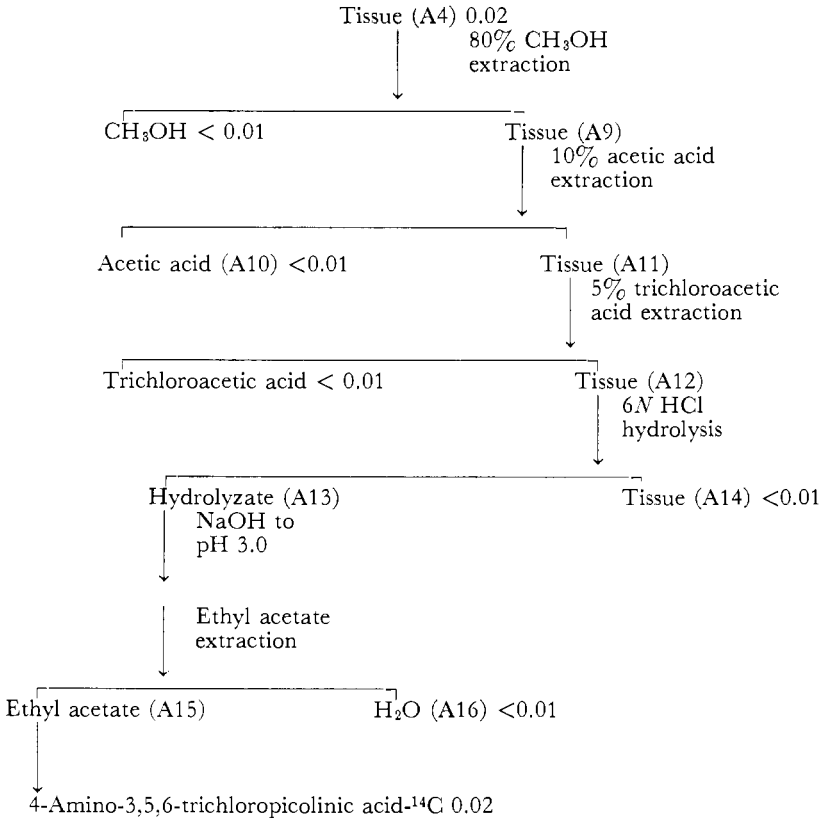

Figure 3. Fractionation procedure applied to extracted leaf and stem tissue containing insoluble radioactive material

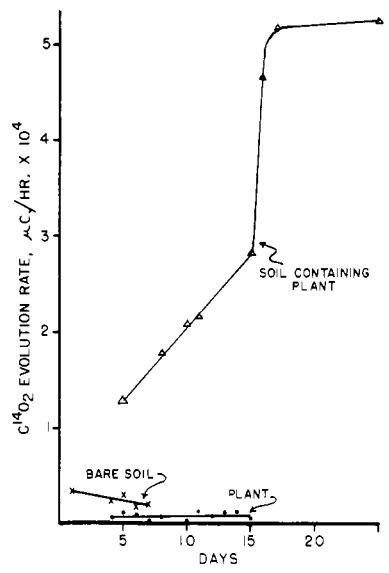

Figure 4. Rates of carbon-^{14}C dioxide evolution

picolinic acid-^{14}C equivalent concentration in each key fraction is given in Figures 1, 2, and 3. Each fraction containing greater than 0.01 p.p.m. Tordon equivalent was investigated by means of paper chromatography to identify provisionally the compounds contributing to the radioactive residue. In each case, the paper chromatograms were counted sufficiently long to make certain that any radioactive entity would be detected at a concentration of 0.01 p.p.m. All the paper chromatography was carried out descending at ambient temperature using Whatman No. 1 paper.

The solvent systems are described in Table I, which lists the fractions investigated, and compares R_f values of the unknown radioactivity to those for known 4-amino-3,5,6-trichloropicolinic acid.

Table I. R_f Values of Unknown Radioactivity in Various Fractions

Fraction[a]	Solvent System[b]	R_f of Unknown	R_f of Tordon
A7	I	0.47[c]	0.47
	II	0.25	0.25
	III	0.42	0.39
	IV	0.36	0.35
B7	I	0.47[c]	0.47
	II	0.26	0.25
	III	0.40	0.39
	IV	0.35	0.35
A15	I	0.44	0.44
	III	0.28	0.29

[a] For identity, see Figures 1, 2, and 3.
[b] Solvent systems. I, 1-butanol saturated with 1.5N ammonia; II, *tert*-amyl alcohol/15N ammonia/water (10/1/5, v./v.); III, benzene/propionic acid/water (2/2/1, v./v.); IV, toluene/propionic acid/water (2/2/1, v./v.).
[c] Unknown and known run on same paper chromatogram; all other cases, run on separate papers but simultaneously.

Discussion

The investigation of the metabolism of Tordon herbicide in soil and cotton took place in two stages: first, detection of carbon-^{14}C dioxide coupled with a study of its rate of evolution; and second, a search for metabolites in the cotton plant. Figure 4 illustrates graphically the manner in which the carbon-^{14}C dioxide evolution rate changes with time.

The expressions in Table II are linear regression equations summarizing the three sets of experimental data. In the case of the experiment involving soil with plant, the data appear to approximate a zero-order rate up to 15 days, at which time the rate of carbon-^{14}C dioxide evolution showed a large increase followed by a leveling off. We have no ready explanation for this phenomenon. The rate of change in the carbon-^{14}C dioxide evolution rate shown by the plant alone was not significant. The rate shown by the bare soil was decreasing significantly with time. If in each of the assumption is made that in each of the experiments the change in carbon-^{14}C dioxide evolution rate over the period of 15 days posttreatment was zero order, then some measure of the relative decomposition rates can be calculated using the regression equations. This is 1:2:26, plant–bare soil–soil with plant. Clearly, the soil containing living plant roots is considerably more active in decomposing Tordon herbicide as measured by carbon-^{14}C dioxide evolution. The contribution made by the cotton plant to decomposition of Tordon was relatively insignificant.

Total plant extracts yielded only Tordon; no other radioactive compounds were detected above a concentration level of 0.01 p.p.m. based on wet weight of the plants.

Table II. Regression Equations Describing $^{14}CO_2$ Evolution Data

Sample	Regression Equation,[a] Microcuries/Hr. × 10^4	Total Evolved $^{14}CO_2$, Microcuries/15 Days × 10^4
Bare soil	0.37 − 0.03 day $S_{y.x}$ = 0.047 S_b = 0.0042	50
Plant	0.067 + 0.0014 day $S_{y.x}$ = 0.057 S_b = 0.0025	24
Soil containing plants	0.54 + 0.15 day $S_{y.x}$ = 0.043 S_b = 0.0013	626

[a] $S_{y.x}$ is standard deviation from regression. S_b is standard deviation of regression coefficient.

The extracted plant tissue retained radioactivity to the extent of 0.02 p.p.m. Not only was this activity retained after initial 80% methanol extraction, but it did not yield to an additional 80% methanol extraction, a 16-hour 10% acetic acid extraction, nor a 2-hour trituration in boiling 5% trichloroacetic acid. Finally, after refluxing with 6*N* hydrochloric acid for 17 hours, radioactive 4-amino-3,5,6-trichloropicolinic acid was isolated; no other radioactive entity down to 0.01 p.p.m. on a fresh plant weight basis was detected. Treatment of plant residues with trichloroacetic acid reagent is reported (*2*) to be a good procedure for removal of most tissue constituents, including polysaccharides, from protein. Since this treatment removed no radioactivity from the solid, extracted residue, it is suggested that the Tordon ultimately isolated from this residue by hydrolysis was combined as an amide with terminal amino groups of protein. The quantity is small but may be significant enough to require a hydrolysis step in any scheme for residue determination.

On a total plant weight basis, the leaf and stem tissue of the cotton plant contained 95% of the total radioactivity present and, of this, 3% was associated with insoluble protein and could not be removed by extraction procedures. The roots contained the remaining 5% of the radioactivity.

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(2) Paech, K., Tracey, M. V., "Modern Methods of Plant Analysis," Vol. **4,** p. 24, Springer-Verlag, Berlin, 1955.

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METABOLISM IN PLANTS

Fate of Radioactive *O,O*-Diethyl O-(2-Isopropyl-4-methylpyrimidin-6-yl) Phosphorothioate on Field-Grown Experimental Crops

JACK W. RALLS, DONNA R. GILMORE, and ANTONI CORTES

Research Foundation, National Canners Association, Berkeley, Calif.

Plants grown in a fenced, controlled, and monitored agricultural plot were sprayed with Diazinon labeled with ^{35}S. The residue level of Diazinon fell rapidly below tolerance levels (0.75 p.p.m.) on all crops studied. There was no evidence of predicted sulfur-containing metabolites at levels above 0.1 p.p.m. on crops treated at recommended dosage. The only metabolite identified from the field samples was oxo-Diazinon at an estimated level of 0.01 to 0.05 p.p.m. Radioactive 2-isopropyl-4-methylpyrimidin-6-ol was isolated from tomatoes 5 days after spraying with pyrimidine ring labeled Diazinon-^{14}C. The present evidence suggests that Diazinon is oxidized rapidly to oxo-Diazinon which is, in turn, hydrolyzed to 2-isopropyl-4-methylpyrimidin-6-ol. The latter compound is metabolized, in part, to carbon dioxide by a pathway which does not appear to involve acetoacetic acid (or its amide).

There are still many unsolved problems of potential public health concern resulting from the use of agricultural chemicals to protect food and fiber crops from ravage by pests. One important problem, as yet not completely understood, is the possible conversion of applied pesticides to other products of potentially increased hazard to food consumers. There are several examples of the identification of transformation products of pesticides which are potentially more toxic than the parent compound. An early example was the demonstration of the conversion of schradan to a *N*-oxide with a 10^5 increase in cholinesterase inhibiting activity (*3*).

This paper reports results of a study of the transformation products formed from Diazinon after spraying on spinach, tomato, and snap bean plants.

Very little information is available on the products formed from Diazinon after application to food crops. One important basic study on the hydrolysis of Diazinon is available from the work of Margot and Gysin (*21*).

The general approach taken in this investigation was to predict the most probable transformation products of Diazinon, prepare these compounds, work out methods for their isolation from the crops of interest, and then learn how to purify, separate, and detect them in small amounts. Once the methodology had been developed, crops were sprayed with radioactive forms of Diazinon and the harvested crops analyzed for the predicted transformation products. Some of the most probable transformation products of Diazinon are shown in Figures 1 to 3.

The cholinesterase inhibiting activity of some of the potential transformation products of Diazinon are shown in Table I. While the cholinesterase inhibiting activity of a compound is not a direct measure of the toxicity of a compound to humans, it is an indication of probable hazard (*21*).

Experimental

Synthesis of Potential Transformation Products of Diazinon. Thiophosphoryl Trichloride, b.p. 119–21° C. (748 torr). Prepared in 74% yield (*15*).

O,O-Diethyl Thiophosphorochloridate, b.p. 54–57° C. (1 torr). Prepared in 76% yield (*10*).

Sodium Diethylphosphate. Prepared by the partial saponification of triethyl phosphate (*19*). Reaction of sodium diethylphosphate with *O,O*-diethyl thiophosphorochloridate (*19*) gave a mixture of tetraethyl pyrophosphate (TEPP) (VI), tetraethyl monothionopyrophosphate (S-TEPP) (VII), and tetraethyl dithionopyrophosphate (SS-TEPP) (VIII).

O,O-diethyl phosphorochloridate, b.p. 57–59° C. (1–2 torr) was prepared in 74% yield (*20*).

The reaction of *O,O*-diethyl phosphorochloridate with 2-isopropyl-4-methylpyrimidin-6-ol (see below for preparation) in refluxing benzene using