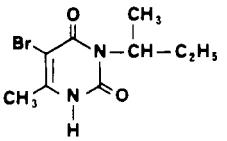
3-Alkoxyuracil Derivatives. 2. Hill Inhibitory Activity

Barry T. Brown, John N. Phillips,* and Barbara M. Rattigan

In a series of 3-alkoxy-5-bromo-6-methyluracils, Hill inhibitory activity, as measured by pI_{50} values determined by using pea chloroplasts, has been shown to be directly proportional to lipophilicity, as measured by log P (1-octanol–water partition coefficient), over a 10 000-fold range of P values. The effects on Hill reaction rate of the 3-alkoxy substituents have been attributed to their interaction with a lipophilic region close to an active center in the chloroplast, while the effects of varying the 1, 5, and 6 substituents have been interpreted in terms of their influence on the interaction of the amide function in the uracil with this hypothetical active site. In contrast to the linear relationship between Hill inhibitory activity and lipophilicity, the corresponding relationship between herbicidal activity and lipophilicity is parabolic in form with the most potent Hill inhibitors herbicidally inactive and with optimum herbicidal effectiveness associated with the C_3 and C_4 alkoxy derivatives.

The mode of action of amide-type herbicides has been interpreted in terms of their interference with the primary photochemical act in photosynthesis (Buchel, 1972; Caseley, 1970; Moreland, 1967). This interference has been demonstrated by their ability to inhibit the Hill reaction, i.e., to inhibit the reduction of an artificial electron acceptor by an irradiated suspension of chloroplasts. The herbicide bromacil (1; 5-bromo-3-*sec*-butyl-6-methyluracil)



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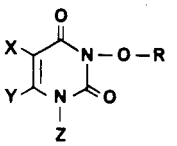

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and a number of related uracils, all of which can be regarded as cyclic amides, have been reported to inhibit the Hill reaction, and their phytotoxicity has been attributed to this ability to block the photosynthetic process (Hilton et. al., 1964; Hoffman, 1972; Hoffman et al., 1964; Kearney and Kaufman, 1975). Certain alkoxy analogues of bromacil have been shown to be herbicidal and to produce phytotoxic symptoms typical of compounds which suppress photosynthesis (Cossey and Phillips, 1980). Accordingly, a study of the relationship between molecular structure and Hill inhibiting activity in a series of such 3-alkoxyuracil derivatives (2) has been undertaken.

EXPERIMENTAL SECTION

Reagents. The 3-alkoxyuracil derivatives were synthesized and characterized and bromacil was purified as described by Cossey and Phillips (1980).

Hill Reaction Assay. Following the procedure of Spencer and Possingham (1960), suspensions of chloroplasts isolated from the leaves of 21-day-old plants of *Pisum sativum* (cv. Victory Freezer) were prepared in a sucrose (0.3 M), potassium chloride (0.01 M), and phosphate buffer (0.05 M, pH 7.3) solution so as to give a chlorophyll concentration of 170 μg/mL as determined by the method of Arnon (1949). One milliliter of this chloroplast suspension and 1.0 mL of an ethanolic solution of the test compound were added to 25.0 mL of Tris buffer (0.1 M, pH 7.3) containing the indicator dye, 2,3',6-trichlorophenolindophenol (TClP: 5×10^{-5} M) as the electron acceptor, the chlorophyll concentration in the assay medium being 6.3 μg/mL (7×10^{-6} M). A 3-mL sample of this solution in a 1-cm cuvette was illuminated for 75 s with white light of saturating intensity. The rate of dye reduction, as determined by absorption measurements at 620 nm, was 250 μmol of TClP (mg of chlorophyll)$^{-1}$ h^{-1}. The activity of the compound as a Hill inhibitor was expressed in terms of its pI_{50} value, i.e., $-\log I_{50}$, where I_{50} was the molar concentration required to decrease the amount of reduction to 50% of that obtained in the absence of the compound.

Phase Distribution Data. Partition coefficients were determined between 1-octanol and aqueous phosphate buffer (10^{-2} M, pH 7.3) at 20 °C, the uracil concentrations being determined by absorption measurments at 270 nm.

RESULTS

Table I (A) records pI_{50} values and 1-octanol–water partition coefficients (P), expressed in logarithmic terms (log P), for a series of 3-alkoxy-5-bromo-6-methyluracil derivatives and for bromacil. Table I (B) records similar data for 3-propoxy- and 3-(octyloxy)uracils, variously substituted in positions 1, 5, and 6 of the heterocyclic nucleus.

Partition data for short and medium chain length alkoxy compounds were determined experimentally, while that for the longer chain length derivatives were determined by extrapolation. Figure 1 shows the linear relationship between log P values and chain length (n) for the unbranched chain 3-alkoxy-5-bromo-6-methyluracil derivatives over the range C_1–C_{10}, from which data for the C_7, C_9, and C_{11}–C_{16} homologues were calculated.

Partition coefficients for the octyloxy derivatives in Table I (B) were calculated by assuming that the experimentally determined Δ log P of 2.62 between the 3-propoxy- and 3-(octyloxy)-5-bromo-6-methyluracils (7) and (17) also applied to other similarly substituted 3-propoxy and 3-octyloxy analogues.

DISCUSSION

3-Alkoxy-Substituted Uracils. The 3-alkoxyuracil derivatives listed in Table I (A) can be regarded as analogues of the herbicide, bromacil (1), in which the 3-*sec*-butyl substituent has been replaced by an alkoxy group containing a straight (**4**; **5**; **7**; **11**; **14–24**), branched (**8**; **12**; **13**), substituted (**6**), or unsaturated (**9**; **10**) alkyl chain. Bromacil has been reported to inhibit the Hill reaction in chloroplast suspensions (pI_{50} = 5.90; Hilton et al., 1964; Hoffman et al., 1964), and this is confirmed by the data recorded in Table I (A) which also shows that the alkoxy analogues behave similarly. While the parent member of the series, the hydroxy compound (**3**), was inactive at the highest concentration tested (3×10^{-3} M), the alkoxy derivatives ranged from inhibitors which were weakly active (pI_{50} = 2.6) to highly potent (pI_{50} = 8.0).

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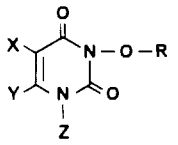
Table I. pI_{50} and Log P Data for 3-Alkoxyuracil Derivatives



part	compd	R	X	Y	Z	log P[a]	pI_{50}[b]
A	3	H	Br	CH_3	H	−1.12	<2.5
	4	CH_3	Br	CH_3	H	−0.30	2.6
	5	C_2H_5	Br	CH_3	H	+0.11	3.9
	6	C_2H_5Cl[e]	Br	CH_3	H	+0.30	4.4
	7	n-C_3H_7	Br	CH_3	H	+0.76	4.9
	8	i-C_3H_7	Br	CH_3	H	+0.45	4.5
	9	C_3H_5[f]	Br	CH_3	H	+0.32	4.3
	10	C_3H_3[g]	Br	CH_3	H	−0.10	3.6
	11	n-C_4H_9	Br	CH_3	H	+1.26	5.4
	12	s-C_4H_9	Br	CH_3	H	+1.11	5.0
	13	i-C_4H_9	Br	CH_3	H	+1.23	5.5
	14	n-C_5H_{11}	Br	CH_3	H	+1.69	5.8
	15	n-C_6H_{13}	Br	CH_3	H	+2.48	6.3
	16	n-C_7H_{15}	Br	CH_3	H	+2.95[c]	6.8
	17	n-C_8H_{17}	Br	CH_3	H	+3.38	7.3
	18	n-C_9H_{19}	Br	CH_3	H	+4.08[c]	7.8
	19	n-$C_{10}H_{21}$	Br	CH_3	H	+4.65	7.9
	20	n-$C_{11}H_{23}$	Br	CH_3	H	+5.21[c]	7.9
	21	n-$C_{12}H_{25}$	Br	CH_3	H	+5.78[c]	8.0
	22	n-$C_{13}H_{27}$	Br	CH_3	H	+6.34[c]	7.9
	23	n-$C_{14}H_{29}$	Br	CH_3	H	+6.91[c]	7.9
	24	n-$C_{16}H_{33}$	Br	CH_3	H	+8.04[c]	7.8
	1			bromacil		+2.11	5.9
B	7	n-C_3H_7	Br	CH_3	H	+0.76	4.9
	25	n-C_3H_7	H	CH_3	H	+0.30	2.5
	26	n-C_3H_7	SCN	CH_3	H	−0.22	3.4
	27	n-C_3H_7	Br	n-C_3H_7	H	+1.73	3.8
	28	n-C_3H_7	Br	CH_3	CH_3	+0.72	<2.5
	17	n-C_8H_{17}	Br	CH_3	H	+3.38	7.3
	29	n-C_8H_{17}	H	CH_3	H	+2.92[d]	5.6
	30	n-C_8H_{17}	SCN	CH_3	H	+2.40[d]	6.9
	31	n-C_8H_{17}	Br	n-C_3H_7	H	+4.35[d]	6.4
	32	n-C_8H_{17}	Br	CH_3	CH_3	+3.34[d]	<3.6

[a] log P = log 1-octanol-water partition coefficient at 20 °C. [b] $pI_{50} = -\log I_{50}$, where I_{50} is the molar concentration of compound giving 50% inhibition of the Hill reaction rate. [c] Determined from the log P vs. n relationship in Figure 1. [d] Calculated by assuming a Δ log P of 2.62 between corresponding octyloxy and propoxy derivatives. [e] C_2H_4Cl = 2-chloroethyl. [f] C_3H_5 = allyl. [g] C_3H_3 = propargyl.

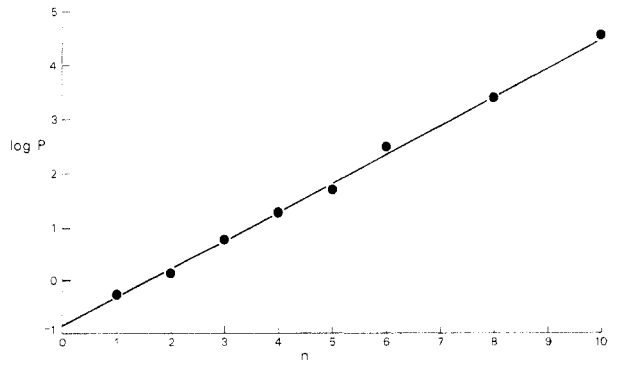


Figure 1. Logarithm of 1-octanol–water partition coefficient (log P) as a function of chain length (n) for unbranched 3-alkoxy-5-bromo-6-methyluracils (●). log P = 0.55n − 0.93 (r = 1.00; s = 0.10).

It is apparent from Table I (A) that increasing the lipophilicity of the alkoxy side chain leads to a progressive increase in the Hill inhibitory activity of the molecule until a limiting level is reached. This is illustrated in Figure 2a

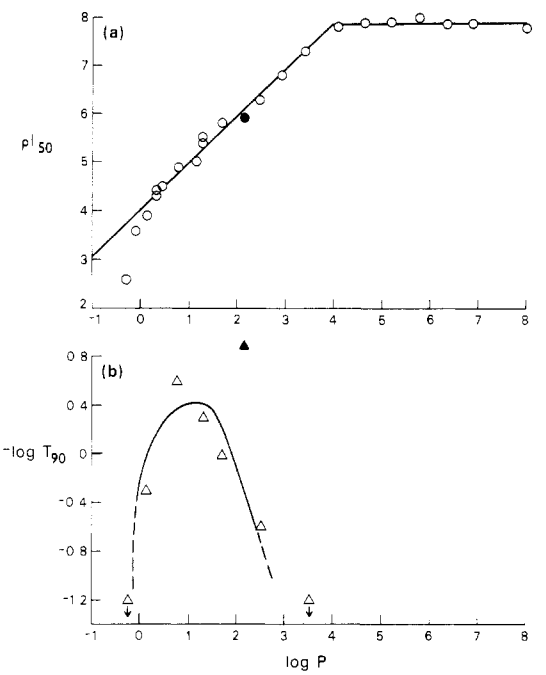


Figure 2. (a) Hill inhibition activity, as measured by pI_{50}, as a function of lipophilicity, as measured by log P, for substituted 3-alkoxyuracil derivatives (○) and bromacil (●). pI_{50} = 0.96 log P + 4.02 (r = 0.99; s = 0.18) (log P = 0 → 4). (b) Herbicidal activity (−log T_{90}) as a function of lipophilicity (log P) for unbranched 3-alkoxy-5-bromo-6-methyluracil derivatives (△) and bromacil (▲).

by a linear function with a positive gradient close to unity (+0.96) in the relationship between pI_{50} and log P for log P values from 0 to 4 and a plateau region for log P values from 4 to 8.

The linear function with a gradient near unity indicates that Hill inhibiting activity is directly proportional to the lipophilicity of the molecule as measured by the 1-octanol–water partition coefficient and is independent of the branched, unbranched, substituted, or unsaturated character of the alkoxy side chain. This suggests that substituents in the 3 position interact with a lipophilic region similar in nature to a liquid hydrocarbon phase and located in the vicinity of the active site. It implies that such substituents are concerned with influencing the affinity of the molecule for the site of action and that the inhibitory activity per molecule at an active site is constant for all members of the series.

The 3-methoxy derivative (4) is anomalous in being less active as a Hill inhibitor than would be predicted from the pI_{50} vs. log P relationship (Figure 2a). This may reflect the inability of this compound, with its short alkyl chain, to interact fully with the lipophilic environment in the vicinity of the receptor site.

The limiting pI_{50} value of 8.0 associated with the plateau region in Figure 2a can be related to the total number of sites in the chloroplast occupied by inhibitor molecules when the Hill reaction rate is reduced by 50%. The concentration of such sites, estimated from the ratio of the limiting inhibitor concentration (10^{-8} M) to the chlorophyll concentration in the assay medium (7×10^{-6} M), corresponds to 1 site per 700 chlorophyll molecules. Assuming the degree of inhibition of the Hill reaction rate to be proportional to the degree of occupancy of sites, then 1 site per 350 chlorophyll molecules would need to be occupied for complete (100%) inhibition of the Hill reaction rate. This value is similar to figures (200–400) deduced from studies with phenylurea and striazine Hill inhibitors (Izawa and Good, 1965), but, as these authors have shown, a

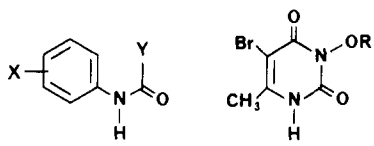


Figure 3. Structural comparison of substituted *N*-phenylamides and 3-alkoxy-5-bromo-6-methyluracils.

significant proportion of the inhibitor molecules absorbed by the chloroplast may be associated with inactive sites.

1,5,6-Substituted Uracil Derivatives. There is insufficient variation in the pattern of the 1-, 5-, and 6-substituted 3-propoxy- and 3-(octyloxy)uracils recorded in Table I (B) to permit the detailed analysis of substituent effects that has been applied to other series of Hill inhibitors (Hansch and Deutsch, 1966). However, comparison of the effect on Hill inhibitory activity of replacing the 1-hydrogen, 5-bromo, or 6-methyl group in compounds **7** or **17** can give some indication of the nature of substituent influences by assuming that activity will tend to be positively correlated with lipophilicity, other factors being equal.

Lack of activity in the N_1-methylated derivatives (**28**; **32**) is unlikely to be due to lipophilic effects, since there is a negligible change in partition behavior ($\Delta \log P = 0.04$) associated with the very significant loss of activity upon methylation ($\Delta pI_{50} > -2.4$ and > -3.7). It may, however, reflect the inability of these compounds to hydrogen bond or readily form a charge transfer complex with a hypothetical receptor site, as has been suggested for other N-substituted phenylamide Hill inhibitors (Hansch, 1969).

Replacement of the 6-methyl group in **7** or **17** by propyl to give compounds **27** and **31** also leads to a significant loss of activity ($\Delta pI_{50} = -1.1$ and -0.9), even though lipophilicity is increased ($\Delta \log P = +0.97$) and differences in the electronic influence of methyl and propyl substituents are trivial. This suggests that the loss of activity is associated with unfavorable steric effects, possibly due to the propyl group interfering more than the methyl group with the interaction of the adjacent amide function with the receptor site.

5-Thiocyanato derivatives (**26**; **31**) are more active inhibitors ($\Delta pI_{50} = +0.9$ and $+1.3$) than the corresponding unsubstituted compounds (**25**; **30**) even though they are less lipophilic ($\Delta \log P = -0.52$). This could be due to the greater electron-withdrawing effect of the thiocyanato group (Hansch et al., 1973). An electron-withdrawing substituent in the 5 position could increase activity by polarizing the amide function so as to enhance its ability to hydrogen bond or form a charge transfer complex with the receptor site. Increased inhibitory activity has been correlated with increased electron-withdrawing character of aryl substituents in various other phenylamide-type Hill inhibitors (Hansch and Deutsch, 1966).

Relationship between Herbicidal Effectiveness and Photosynthetic Inhibitory Activity. The preemergent herbicidal activities of bromacil and of unbranched 3-alkoxy-5-bromo-6-methyluracil derivatives against a typical sensitive species, ryegrass, (*Lolium perenne* cv. New Zealand) have been assessed in terms of the lowest application rate (T_{90}) giving less than 10% plant survival 6 weeks after sowing (Cossey and Phillips, 1980). The relationship between $-\log T_{90}$ and $\log P$ is parabolic in form (Figure 2b), with optimum herbicidal effectiveness associated with $\log P$ values of ~ 1.0 corresponding to the C_3 and C_4 alkoxy derivatives. In contrast, photosynthetic inhibitory activity increases with increasing chain length up to $n = 9$, so that the most potent photosynthetic inhibitors (compounds **15**–**24**, $pI_{50} \sim 7.9$) are nonherbicidal ($T_{90} > 16$ kg ha^{-1}). Thus the 3-octyloxy derivative (**17**) ($pI_{50} = 7.8$) is inactive against ryegrass at 16 kg ha^{-1}, while the 3-propoxy analogue (**7**) ($pI_{50} = 4.9$) is phytotoxic at 0.125 kg ha^{-1}. Lack of activity among these highly lipophilic derivatives may be due to an unfavorable distribution within the plant so that insufficient molecules reach the active centers in the chloroplast.

The observed Hill inhibitory activity for bromacil ($pI_{50} = 5.9$) agrees with that calculated ($pI_{50} = 6.0$) from the pI_{50} vs. $\log P$ relationship for the alkoxyuracils (Figure 2a), suggesting that both 3-alkyl- and 3-alkoxyuracil derivatives act at the same photosynthetic center. However, the herbicidal activity of bromacil appears to be significantly greater than that predicted from the $-\log T_{90}$ vs. $\log P$ relationship for the alkoxy series (Figure 2b). This could be explained by the 3-alkoxy derivatives being more readily degraded than analogous 3-alkyl compounds, e.g., bromacil because of the nitrogen–oxygen bond being potentially more labile than the nitrogen–carbon link.

Nature of the Receptor Site. Quantitative structure–activity correlations in various anilide, phenylcarbamate, and phenylurea Hill inhibitors, coupled with a consideration of their common structural features, have led to a proposed mode of action in which Hansch (1969) suggested that inhibition of the Hill reaction by phenylamide derivatives arose from a charge transfer interaction between the amide moiety in the inhibitor molecule and an amide receptor site located near a hydrophobic zone of the protein. At the biochemical level it is believed that such inhibitors act by blocking electron flow between the primary (Q) and secondary (B) acceptor of photosystem II (Pfister and Arntzen, 1979; Trebst and Draber, 1979).

The results reported here are broadly consistent with the Hansch concept, in that the 3-alkoxy substituents could be regarded as interacting with a lipophilic region corresponding to the hydrophobic zone and the N-1 amide function as interacting with the receptor site. In the *N*-phenylamide (Xϕ–NH–CO–Y) series it has been shown (Hansch and Deutsch, 1966) that it is the X substituent on the phenyl ring, and not the Y group, that is primarily involved in hydrophobic bonding. However, with the uracil derivatives it is the 3-alkoxy substituent that is implicated, and, relative to the free amide function, this substituent corresponds spatially to that of the Y group in the phenylamides (Figure 3). This may appear somewhat anomalous in that photosystem II inhibitors have been shown to act on a common target (Van Assche, 1980), but it is not inconsistent with recent suggestions that different classes of PSII inhibitors interact with different areas of the hydrophobic domain surrounding the active site (Pfister and Arntzen, 1979; Trebst and Draber, 1979).

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Determination of the Insecticide/Acaricide Formetanate in Fresh Fruit by Reversed-Phase Liquid Chromatography

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Formetanate [*m*-[[(dimethylamino)methylene]amino]phenyl methylcarbamate] was extracted from fresh samples of oranges, apples, plums, peaches, and pears with acidified acetonitrile. An aliquot of the filtered extract was evaporated to an aqueous residue, which was cleaned up by alternate acidic and basic aqueous–organic partitions. The final residue was dissolved in distilled water for analysis by liquid chromatography on an RP-18 reversed-phase column with a mobile phase consisting of 35% acetonitrile in 0.01 M ammonium phosphate, monobasic (pH 8.0), and UV detection at 254 nm. Average recoveries at ≥0.5 ppm were over 80%. At the 0.1-ppm level average recoveries were generally 70% or higher. Minimum detectable levels were estimated to be ~0.02–0.05 ppm in the fruits studied.

Formetanate [*m*-[[(dimethylamino)methylene]amino]-phenyl methylcarbamate], in the form of its hydrochloride salt, is used as an insecticide/acaricide for control of spider mites, rust mites, certain aphids, thrips, lygus bugs, leaf hoppers, slugs, and snails on a variety of orchard fruit (Jenny and Kossmann, 1978). Interest in this compound has recently arisen as a result of questionable toxicology studies which were used to secure its registration for use in the United States and Canada. No data have been reported up to now on residue levels of this pesticide in the food supply, in particular, orchard fruits such as citrus, apples, peaches, pears, and plums. In the past, analytical techniques for formetanate residues involved hydrolysis of the pesticide to yield m-aminophenol which was diazotized and coupled with *N*-(1-naphthalene)diamine dihydrochloride to produce a reddish blue azo dye which could be quantitated by colorimetry (Jenny and Kossman, 1978). An improved method involving gas chromatography (GC) was also devised by Jenny and Kossman (1978) which made use of hydrolysis of the pesticide to *m*-aminophenol followed by bromination which yielded 2,4,6-tribromophenol. This product was then determined by GC with electron-capture detection. Both of these methods are indirect, requiring chemical reactions and the measurement of a derivative rather than the parent compound. Other carbamates have been directly determined in foods by LC (Lawrence, 1977; Lawrence and Leduc, 1977, 1978; Nelsen and Cook, 1979; Robinson and Chapman, 1980; Thean et al. 1978); however, no applications to formetanate were included. We have developed a direct method for the analysis of formetanate which makes use of reversed-phase liquid chromatography (LC) and UV-absorbance detection. As a result, the technique is more rapid and involves less sample manipulation than the previously described colorimetric or GC methods.

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The method has been applied to a limited survey of fruits from various regions of Canada.

EXPERIMENTAL SECTION

Reagents. All organic solvents used for sample extractions, cleanup, and liquid chromatography were distilled-in-glass grade. The stock solution of formetantate hydrochloride was prepared in acetonitrile at a concentration of 1.0 mg/mL. Dilutions of this for spiking purposes were made with acetonitrile. Chromatography solutions were prepared by appropriately diluting the the stock with distilled deionized water. These were stored in a refrigerator when not in use and were stable for at least 1 week. Chromatography standards were never prepared in the LC mobile phase due to the degradation of the pesticide at pH 8–9, which caused losses of ~20% after 1 day.

The fruits were purchased in five different regions of Canada and consisted of both domestic and imported goods. Recovery studies were carried out on locally purchased samples of oranges, plums, and apples.

Sample Extraction. Twenty-five grams of homogenized fruit was blended in a Sorvall Omnimixer for 3 min at medium speed with 70 mL of acetonitrile containing 0.5% concentrated HCl. The mixture was filtered with suction through a medium-porosity sintered glass funnel and the filtrate collected in a 100-mL volumetric flask. The residue in the funnel was rinsed with a small volume of acetonitrile and the volume of the total filtrate adjusted to 100 mL.

Cleanup. A 20-mL aliquot of the filtrate (equivalent to 5.0 g of sample) was transferred to a 100-mL round-bottom flask and evaporated under vacuum at 30 °C to an aqueous residue (~2 mL). To this was added 3 mL of H_2O and the contents were quantitatively transferred to a 125-mL separatory funnel containing 5 mL of 0.2 N H_2SO_4 and 10 mL of methylene chloride. The funnel was shaken by hand for 1 min and the layers were permitted to separate. The methylene chloride layer was discarded. Following this 10 mL of saturated sodium chloride solution

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