radioactivity found in the heavier RIR/BPR chickens. Miller et al. (1976) reported higher tissue residue levels in the WL chickens. The delayed sampling of the tissues (14 days after a single oral dose) may explain this apparent conflict. Recoveries of [^{14}C]diflubenzuron in the eggs of the respective chickens confirm that WL chickens excrete about twice the amount of diflubenzuron in their eggs as RIR/BPR chickens. Higher metabolism rates by the WL chickens seemed to indicate this as a possible explanation. However, the presence of only unmetabolized diflubenzuron in the eggs makes any hypothesis as to an explanation based on metabolism difficult.

Comparison of the liver microsomal metabolism of diflubenzuron between these two types of chickens was made to further characterize the metabolic differences on a subcellular level. It was found that the microsomal metabolism differences were less than in vivo metabolism differences.

Chicken liver microsomal induction via phenobarbital has been demonstrated by Stephen et al. (1971) and Powis et al. (1976). The present study demonstrated liver microsomal induction after phenobarbital pretreatment, with increased liver to body weight ratios and increased amount of cytochrome P-450. All of these studies have found lower P-450 levels in chickens than those reported in rats. Induction of aldrin epoxidase levels in both WL and RIR/BPR chicken livers did not result in enhanced diflubenzuron metabolism.

Chicken liver microsomal metabolism of [^{14}C]diflubenzuron was very low in both types of hens, with or without phenobarbital pretreatment. Chromatographic characterization of organic extracts after microsomal incubations indicated some slight differences in WL and RIR/BPR metabolites. Both types of hens produced cleavage metabolites after microsomal incubation. Mixed-function oxidase enzymes do not seem to be primarily involved in diflubenzuron metabolism in chicken microsomes.

The results of this study of diflubenzuron metabolism by WL and RIR/BPR chickens indicate slight differences in metabolite formation. In no instance does the breed-related metabolism difference reach as much as 2-fold. Metabolism differences of WL and RIR/BPR chickens are not of the same order of magnitude as the egg residue level differences; therefore, they do not seem to account for the diflubenzuron egg residue level difference.

LITERATURE CITED

Gillette, J. R. In "Fundamentals of Drug Metabolism and Drug Disposition"; LaDu, B. N.; Mandel, H. G.; Nay, E. L., Eds.; Waverly Press: Baltimore, MD, 1976; pp 400–418.
Ivie, G. W. *J. Agric. Food Chem.* **1978**, *26*, 81.
Lowry, O. H.; Rosenbrough, N. J.; Farr, H. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265.
Metcalf, R. L.; Lu, P. Y.; Bowlus, S. *J. Agric. Food Chem.* **1975**, *23*, 359.
Miller, R. W. *J. Econ. Entomol.* **1974**, *67*, 697.
Miller, R. W.; Corley, C.; Hill, H. R. *J. Econ. Entomol.* **1975**, *68*, 181.
Miller, R. W.; Corley, C.; Shufelt, S. R. *J. Econ. Entomol.* **1976**, *69*, 741.
Omura, T.; Sato, R. *J. Biol. Chem.* **1964**, *239*, 2370.
Opdycke, J. C.; Menzer, R. E. *J. Toxicol. Environ. Health*, **1982**, in press.
Opdycke, J. C.; Miller, R. W.; Menzer, R. E. *J. Agric. Food Chem.* **1982**, preceding paper in this issue.
Powis, G.; Drummond, A. H.; MacIntyre, D. E.; Jondorf, W. R. *Xenobiotica* **1976**, *6*, 69.
Stephen, B. J.; Garlich, J. D.; Guthrie, F. E. *Bull. Environ. Contam. Toxicol.* **1971**, *5*, 569.
VanDaalen, J. J.; Meltzer, J.; Mulder, R.; Wellinga, K. *Naturwissenschaften* **1972**, *7*, 312.
Willems, H. G. M.; Overmars, H.; Scherpenisse, P.; DeLange, N.; Post, L. C. *Xenobiotica* **1980**, *10*, 103.
Wright, J. E. *J. Econ. Entomol.* **1974**, *67*, 746.
Wright, J. E. *J. Econ. Entomol.* **1975**, *68*, 322.
Wright, J. E.; Spates, G. E. *J. Econ. Entomol.* **1976**, *69*, 365.

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Pesticide Residues in Soil. 1. Gas Chromatographic Determination of Vinclozolin

Raffaella Boccelli,* G. Pietro Molinari, and Attilio Del Re

Vinclozolin [3-(3,5-dichlorophenyl)-5-ethenyl-5-methyloxazolidine-2,4-dione] is a possible contaminant of soil as a result of fungicide treatments. It is taken up by benzene with pyrophosphate for analysis; the extracts are analyzed by gas chromatography with an electron-capture detector, without cleanup. For concentrations from 0.01 to 10 ppm, the recovery from soil is good (93 ± 2%). The detection limit is 0.001 ppm.

In fruit growing we cannot do without pesticides: most treatments are directed to the aerial part of plants, but it is not possible to avoid the fall of pesticides on soil, often also in large quantity. Moreover, rains, dews, and wind wash away and remove physically the residues from leaves and fruits to the soil, where both the active ingredient and some transformation products can accumulate. The presence of a pesticide can influence chemical and microbiological properties of the soil (Martin, 1963; Siegel, 1975; Wainwright, 1977), but the study of interactions between pesticides and soil is only possible if there are analytical methods able to assess the quantities of pesticides present.

It seemed to us interesting to take Vinclozolin into consideration: Vinclozolin is the active ingredient of the fungicide sold as Ronilan, with a fair gray-mold capability (Bolay et al., 1976; Piglionica and Ferrara, 1979; Di Giusto et al., 1980; Gullino et al., 1980; Carniel and Micolini, 1980), extensively used in the vineyard, in fruit and vegetable

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Table I. Physical and Chemical Data on Soils in the Recovery Tests

soil source	pH (1:2.5)	clay, %	sand, %	silt, %	organic matter, %	nitrogen, %	K_2O av,[a] %	P_2O_5 av,[a] %
Piacenza (Italy)	7.8	24	41	35	3.8	0.250	0.0226	0.0033
Brazzaville (Congo)	5.2	23	67.5	9.5	2.9	0.105	0.001	trail
Mareto (Piacenza, Italy)	5.3	21	49	30	12	0.52	0.041	0.0019

[a] Available.

Table II. Recovery of Vinclozolin from Fortified Soils

soil source	fortification level, ppm[a]	recovered Vinclozolin ppm	recovered Vinclozolin %	% av recoveries ± SD[b]
Piacenza[c]	0.01	0		
Piacenza[c]	0.01007	0.0100	99	
Piacenza[c]	0.01007	0.0120	119	104.6 ± 12.8
Piacenza[c]	0.01007	0.0096	96	
Piacenza[c]	0.1007	0.0981	97	
Piacenza[c]	0.1007	0.1111	110	
Piacenza[c]	0.1007	0.1078	107	
Piacenza[c]	0.1007	0.1030	102	
Piacenza[c]	0.1007	0.0921	91	97.6 ± 7.3
Piacenza[c]	0.1007	0.0910	90	
Piacenza[c]	0.1007	0.0921	91	
Piacenza[c]	0.1007	0.0945	94	
Piacenza[c]	0.1007	0.0948	94	
Piacenza[c]	1.007	0.886	88	
Piacenza[c]	1.007	0.859	85	87.3 ± 1.7
Piacenza[c]	1.007	0.892	88	
Piacenza[c]	5.11	4.79	94	
Piacenza[c]	5.11	4.97	97	93.5 ± 3.8
Piacenza[c]	5.11	4.58	90	
Piacenza[c]	10.21	8.70	85	
Piacenza[c]	10.21	9.21	90	88.7 ± 3.1
Piacenza[c]	10.21	9.27	91	
Brazzaville[d]	5.04	4.80	95	
Brazzaville[d]	5.04	4.53	90	91.9 ± 2.9
Brazzaville[d]	5.04	4.56	90	
Mareto[e]	5.04	3.96	79	
Mareto[e]	5.04	4.29	85	81.2 ± 3.5
Mareto[e]	5.04	4.02	80	

[a] Aliquots of 0.5 mL of Vinclozolin as a benzoic solution at 0.5, 5.0, 50.5, 255, and 510 μg/mL have been added to 25 g of dried, sieved soil. [b] Average recovery percent ± mean standard deviation = $\bar{X} \pm S_{\bar{X}} = 93 \pm 2$. [c] Piacenza, Italy. [d] Brazzaville, Congo. [e] Piacenza, Italy.

cultivation, in the greenhouse and in open fields.

There are not much data about the levels of Vinclozolin residues at present, and those that are restricted to grapes (Bencivenga et al., 1980), must, wine (Bolay et al., 1976; Molinari and Del Re, 1978; Barbina Taccheo et al., 1980), strawberries (Zanini et al., 1980; Flori et al., 1980), and peppers (Ronco et al., 1981). The aim of the present paper is to propose an analytical method by which it is possible to extract Vinclozolin residues from soil with a good yield and to analyze the extracts by gas chromatography (GLC) without preliminary cleanup. Only for residues below 0.1 ppm is it necessary to concentrate extracts at least twice before GLC analysis.

EXPERIMENTAL SECTION

Apparatus. The following were used: Dubnoff's shaking water bath at a temperature of 18 °C; centrifuge for 300-mL bottles; DANI gas chromatograph, Model 3600, with either (1) Ni^{63} electron capture detector (ECD) and glass column [2 m × 2 mm i.d., packed with OV-11 at 5% on Chromosorb W, AW-DMCS, 80–100 mesh, column temperature (isothermal) 200 °C, and carrier gas (N_2 flow rate 60 mL/min)] or (2) ECD and flame ionization (double) detector [Model ECD-FID 68/51 Tandem (DANI)] and glass column [2 m × 2 mm i.d., packed with OV-11 at 2% and QF-1 at 3% on Chromosorb W, AW-DMCS, 80–100 mesh, column temperature (isothermal) 190 °C, and carrier gas (N_2 flow rate 60 mL/min)]; 300-mL glass bottles with screw caps supplied with Teflon-lined silicone septums (Pyrex); rotary vacuum evaporator.

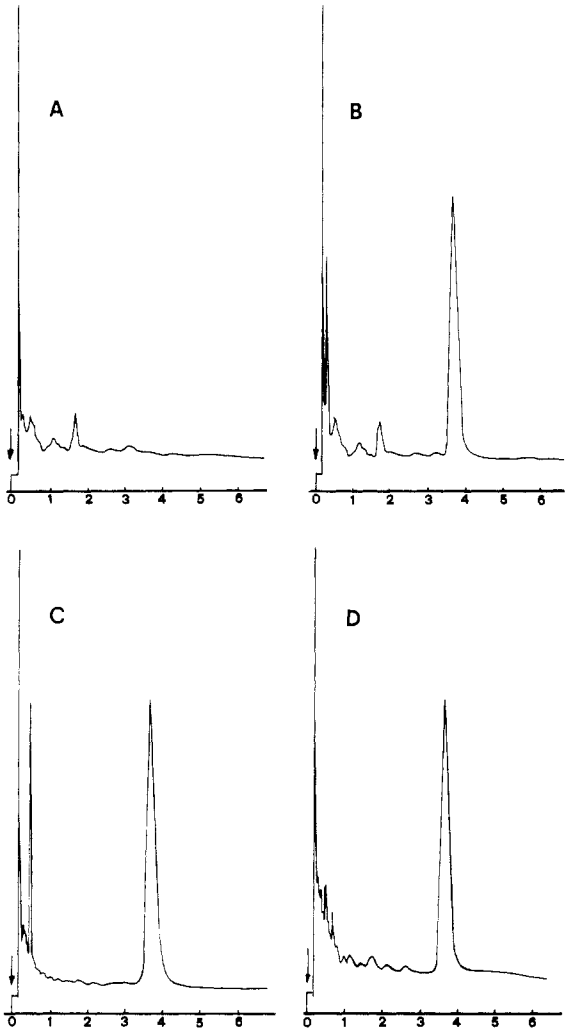

Figure 1. GLC chromatograms, ECD response, of (A) an extract of nontreated soil, (B) the same soil of (A) spiked with 7.5 μg of Vinclozolin (as 0.5 mL of benzene solution at 15.0 μg/mL) in 25 g of soil before extraction, (C) a standard mixture of 3,5-dichloroaniline (DCA) 3.94 ng) and Vinclozolin (0.2 ng), and (D) an extract of soil field treated with Ronilan (sample 5 of Table III), on OV-11/QF-1 column packing at 190 °C with a carrier gas (N_2) flow rate of 60 mL/min.

Reagents. The following were used: Sodium pyrophosphate, 0.1 M, in water; pure benzene (gas chromatographic purity 99.5%); Vinclozolin [3-(3,5-dichlorophenyl)-5-ethenyl-5-methyloxazolidine-2,4-dione] extracted from Ronilan, recrystallized twice in hexane to a sharp mp of 108 °C [108 °C (Martin and Worthing, 1977)], and characterized by GLC/MS (Selva et al., 1981); standard solutions of Vinclozolin in benzene (0.50, 5.00, 50.50, 255.0, and 510 μg/mL), prepared by diluition of a more concentrated standard in the same solvent; pure anhydrous so-

Table III. Vinclozolin Residues in Soils from Vineyard Field Treated with Ronilan

sample	no. of field treatments	time between last treatment and sampling, months	soil source	recovered Vinclozolin, ppm: by described method	recovered Vinclozolin, ppm: by DCA method
1	4	5	Pianello[b]	0.06	1.00
2	4	5	Pianello[b]	0.24	1.73
3	4	6	Pianello[b]	0.10	0.13
4	4	6	Pianello[b]	0.17	1.66
5	4	6	Pianello[b]	0.30	1.13
6	4	6	Pianello[b]	0.01	1.32
7	4	7	Pianello[b]	0.30	2.11
8	4	7	Pianello[b]	0.90	2.65
9	4	7	Pianello[b]	0.18	1.20
10	4	7	Pianello[b]	2.10	1.15
11	4	8	Pianello[b]	0.004	1.34
12	4	8	Pianello[b]	0.06	0.25
13	4	8	Pianello[b]	0.04	1.31
14	4	8	Pianello[b]	0.03	0.035
15	4	8	Pianello[b]	0.02	1.89
16	4	8	Pianello[b]	0.02	0.94
17	4	9	Pianello[b]	0.03	0.83
18	4	9	Pianello[b]	0.02	1.54
19	4	9	Pianello[b]	0.025	1.22
20	4	9	Pianello[b]	0.02	0.69
21	3	5	Voghera[c]	ND[a]	
22	0		Retorbido[c]	ND	

[a] ND = none detected; residue < 0.001 ppm. [b] Piacenza, Italy. [c] Pavia, Italy.

dium sulfate. All reagents have to be analyzed for the absence of artifacts in the GLC.

Sample Preparation and Extraction. Soil, air-dried just before the analysis, is ground and screened through a 2-mm (10-mesh) sieve. A 25-g aliquot is transferred into a 300-mL glass bottle with a screw cap; 50 mL of $Na_4P_2O_7$, 0.1 M (Kononova, 1966), is added and then 75.0 mL of benzene. The stoppered bottles are shaken for 30 min on a Dubnoff's water bath, capped to exclude light, and centrifuged at 1500 rpm for 5 min. The benzene is recovered and filtered on a fluted paper covered with 5 g of anhydrous sodium sulfate.

For concentrations less than 0.1 ppm, a 25-mL aliquot of extract is exactly measured, concentrated by using a rotary vacuum evaporator, taken up with benzene, transferred into a graduated flask, and diluted to 10 mL with benzene. For concentrations greater than 0.1 ppm, the extract is injected directly.

Gas Chromatography. Both columns were suitable for the purpose; the retention times for Vinclozolin and DCA were respectively 5 and 0.8 min on column OV-11; 3.65 and 0.45 min on column OV-11/QF-1. Residue concentrations have been calculated by comparing the Vinclozolin peak area of the sample with a calibration curve obtained by injecting 1-μL aliquots of standard solutions at suitable concentrations in benzene. The chromatograms on the OV-11/QF-1 column of a soil-free fungicide, of the same soil fortified with Vinclozolin, of a standard mixture of Vinclozolin and 3,5-dichloroaniline (DCA), and of a soil coming from a treated vineyard are shown in Figure 1.

RESULTS AND DISCUSSION

Recovery tests have been made on three vastly different soil types, representative of extreme pedological situations. The basic parameters of these soils obtained by the methods of SISS (1976) and Olsen and Cole (1954) are given in Table I.

The analysis has been done just after fortification of soils. Percent recoveries of Vinclozolin and averages with standard deviation are shown in Table II.

Table III presents some results of a study on Vinclozolin contamination in soil of vineyards treated with Ronilan. The sampling and analysis began 5 months after the last treatment. The results obtained with this method are compared with the results obtained by a method based on alcaline hydrolysis to 3,5-dichloroaniline (DCA) and its steam distillation and determination by spectrophotometry (Zweig, 1974; Barbina Taccheo et al., 1978).

The proposed method has given satisfactory results with acidic or basic soils with organic matter levels from 2.9 to 12%. In the range of concentrations from 0.01 to 10.2 ppm of Vinclozolin, the grand mean and standard deviation of recoveries are 93 ± 2%. The detection limit of the method is 0.001 ppm. The results of analysis of vineyard-treated soils with the proposed method are lower because the alkaline hydrolysis method (DCA method) is not specific for Vinclozolin, but Vinclozolin metabolites and degradation products are detected in addition to the parent compound (Otto, 1980). The proposed method is fast, without cleanup for levels over 0.1 ppm, and specific for Vinclozolin residues.

LITERATURE CITED

Barbina Taccheo, M.; Baruzzini, L.; Spessotto, C. "Atti Giornate Fitopatologiche"; CLUEB: Bologna, Italy, 1978; Vol. I, p 31.
Barbina Taccheo, M.; Baruzzini, L.; Spessotto, C. "Atti Giornate Fitopatologiche"; CLUEB: Bologna, Italy, 1980; Vol. I, p 27.
Bencivenga, B.; Pallotti, G.; Simonetti, T.; de Vincenzi, S.; Pasquazi, G. *Ind. Aliment. (Pinerolo, Italy)* **1980**, *178*, 936.
Bolay, A.; Aerny, J.; Crettenand, J.; Gnaegi, F.; Pezet, R.; Schopfer, J. F. *Rev. Suisse Vitic., Arboric., Hortic.* **1976**, *8*, 195.
Carniel, P. L.; Micolini, S. "Atti Giornate Fitopatologiche"; CLUEB: Bologna, Italy, 1980; Vol. II, p 587.
Di Giusto, R.; Avonda, S.; Ferrari, G.; Molinari, V.; Salgarollo, V.; Tesi, D. "Atti Giornate Fitopatologiche"; CLUEB: Bologna, Italy, 1980; Vol. II, p 529.
Flori, P.; Brandolini, V.; Stanzani, R.; Musacci, P. "Atti Giornate Fitopatologiche"; CLUEB: Bologna, Italy, 1980; Vol. I, p 19.
Gullino, M. L.; Di Punzio, F.; Mancini, G.; Garibaldi, A. "Atti Giornate Fitopatologiche"; CLUEB: Bologna, Italy, 1980; Vol. II, p 577.
Kononova, M. M. "Soil Organic Matter", 2nd ed.; Pergamon Press: Oxford, 1966.
Martin, H.; Worthing, C. R. "Pesticide Manual", 5th ed.; British Crop Protection Council: London, England, 1977; p 535.
Martin, J. P. *Residue Rev.* **1963**, *4*, 96.
Molinari, G. P.; Del Re, A. *Chim. Ind. (Milan)* **1978**, *60*, 705.
Olsen, S. R.; Cole, V. C. *U.S., Dep. Agric., Circ.* **1954**, *No. 939.*

Otto, S. *Atti Simp. Chim. Anticrittogamici Piacenza* **1980**, *1*, 109.
Piglionica, V.; Ferrara, G. *Dif. Piante* **1979**, *4*, 207.
Ronco, C.; Barberis, E.; Sapetti, C. *Atti Simp. Chim. Antiparassitari Piacenza* **1981**, *2*, 98.
Selva, A.; Marchini, G. F.; Griffini, A.; Molinari, G. P. *Atti Simp. Chim. Antiparassitari Piacenza* **1981**, *2*, 139.
Siegel, M. R. *Phytopathology* **1975**, *65*, 219.
SISS (Società Italiana della Scienza del Suolo) "Metodi Normalizzati di analisi del suolo"; Tipografia G. Capponi: Firenze, Italy, 1976.
Wainwright, M. *Z. Pflanzenernaehr. Bodenkd.* **1977**, *140*, 587.
Zanini, E.; Barberis, E.; Ronco, C. *J. Agric. Food Chem.* **1980**, *28*, 464.
Zweig, G. "Analytical Methods for Pesticide, Plant Growth Regulators, and Food Additives"; Academic Press: New York, 1964; Vol. IV.

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A Series of Pyromellitic Diimides That Improve the Efficiency of Rumen Fermentation

Bruce O. Linn,* Lynn M. Paege, Patrick J. Doherty, Richard J. Bochis, Frank S. Waksmunski, Peter Kulsa, and Michael H. Fisher

A number of pyromellitic diimides were prepared and found to alter rumen fermentation in a potentially beneficial way. In vitro rumen test procedures and chemical syntheses for preparation of novel unsymmetrical N-substituted pyromellitic diimides were developed. Structure–activity investigations showed that a variety of N substituents could be tolerated while retaining high activity. The most potent compounds in the series had ID_{50} values for methane suppression of approximately 1 μg/mL and blocked methane production completely at higher levels. Volatile fatty acid composition was shifted from acetic to propionic and butyric acids. Two compounds, pyromellitic diimide and pyromellitic *N*-(2-hydroxyethyl)diimide, have been selected for further study in sheep and cattle.

The rumen, in ruminating animals, provides an ecosystem in which microorganisms, living at a pH between 5.5 and 7.0 in an anaerobic environment, can metabolize incoming materials into nutrients to be used by the host. The principal end products of carbohydrate metabolism in this system are volatile fatty acids (VFA's) such as acetic acid, propionic acid, and butyric acid, which are absorbed by the host and the gases carbon dioxide and methane. Methane is formed by the reduction of carbon dioxide by hydrogen. An outline of the principal pathways of carbohydrate metabolism is shown in Scheme I [adapted from Hungate (1966) and Leng (1970)]. Methane is expelled.

A typical rumen fermentation may be approximated by the equation (Wolin, 1974)

$$57.5C_6H_{12}O_6 \rightarrow 65CH_3COOH + 20CH_3CH_2COOH + 15CH_3CH_2CH_2COOH + 60CO_2 + 35CH_4 + 25H_2O \quad (1)$$

If one considers energy utilization and conservation via glycolysis, the following generalization applies (Hungate, 1966):

$$\underset{672\ \text{kcal/mol}}{C_6H_{12}O_6} \rightarrow \underset{420\ \text{kcal/mol}}{CH_3COOH} + \underset{734\ \text{kcal/mol}}{CH_3CH_2COOH} + \underset{524\ \text{kcal/mol}}{CH_3CH_2CH_2COOH} + CH_4 \quad (2)$$

Thus, the elevation of the levels of propionic and butyric acids, with concomitant reduction of acetic acid, should improve the energetics of rumen fermentation and hence enhance feed efficiency in ruminant animals (Hungate, 1966; Chalupa, 1977). Any reduction in the production of methane, a waste product, should result in energy conservation.

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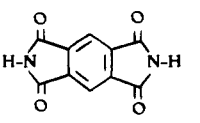
There have been a number of publications describing the enhancement of feed efficiency in sheep and cattle by monensin (Davis and Erhart, 1976; Perry et al., 1976; Potter et al., 1976a,b; Raun et al., 1976). Part of this improvement was attributed to a favorable alteration of rumen energetics by elevation of propionic acid and reduction of acetic acid production (Richardson et al., 1976).

BIOLOGICAL EVALUATION

An approach taken in these laboratories was to search for compounds that would suppress methane production in rumen fluid while lowering acetic acid and raising propionic acid levels. Such compounds would be expected to improve feed efficiency in cattle and sheep. A rapid, high-capacity, in vitro, batch-type rumen fermentation system was developed, utilizing strained rumen fluid and a high-energy feed ration (see Experimental Section, Test Procedures). Selected compounds were tested at 250 μg/mL. Those compounds showing better than 70% methane inhibition, and a shift of acetic acid to propionic, butyric, or valeric acids were retested at lower levels. The most active compounds were titrated in an 18-h assay in order to determine the dose at which methane production was inhibited to 50% of control (ID_{50}) and the VFA composition at this level.

Pyromellitic diimide (1) was highly active in these procedures and a structure–activity investigation was undertaken.



I

CHEMISTRY

The synthesis of pyromellitic symmetrically N,N′-disubstituted diimides (II) from pyromellitic dianhydride

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