important safeguard of public health.

Registry No. Carbofuran, 1563-66-2; chlorozolinate, 72391-46-9; parathion methyl, 298-00-0.

LITERATURE CITED

European Directory of Agrochemical Products. III. Insecticides and Acaricides; The Royal Society of Chemistry, University of Nottingham: England, 1984.
FAO/WHO 1968 Evaluation of Some Pesticide Residue in Food, 1969, pp 241-255.
FAO/WHO 1976 Evaluation of Some Pesticide Residue in Food, 1977, pp 135-186.
FAO/WHO Pesticide Residues in Food, 1979, 1980, p 71.
Italian Health Department Act (Oct 23, 1985) *Gazz. Uffic. Repub. Ital.* **1985**, *8*.
Meloni, M.; Pirisi, F. M.; Cabras, P.; Cabitza, F. *J. Agric. Food Chem.* **1984**, *32*, 183-185.
Mollhoff, E. *Pflanzenschutz-Nachr. Bayer (Ger. Ed.)* **1968**, *21*, 331-358.

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O-Acylation as a Novel Conjugation Pathway for Cinmethylin in Rats

Philip W. Lee,* Michael D. Woodward, and Stephen M. Stearns

A complex degradation pattern of [*phenyl*-^{14}C]cinmethylin (1) in laboratory rats following oral administration has been reported earlier. In addition to the undegraded 1, 10 metabolites were detected. In the urinary organic extractable fraction, two minor metabolites (each accounting for less than 1% of the administered radioactivity) were identified as *o*-(acetoxymethyl)benzoic acid and 9-(acetoxymethyl)-α-carboxycinmethylin. They were the corresponding *O*-acetyl analogues of *o*-(hydroxymethyl)benzoic acid and 9-hydroxy-α-carboxycinmethylin, the principal metabolites of 1. A detailed description of the isolation and identification of these two novel O-acylation conjugates is presented.

The metabolic fate of [*phenyl*-^{14}C]cinmethylin (1) [Cinch herbicide, 7-oxabicyclo[2.2.1]heptane, 1-methyl-4-(1-methylethyl)-2-[(2-methylphenyl)methoxy]-, *exo*], a novel cineole herbicide, in laboratory rats has been reported (Lee et al., 1986). Following a single oral dose, the major route of elimination was via urinary excretion. Approximately 75–85% of the administered radioactivity was eliminated during the initial 48-h postdosing. A complex degradation pattern of 1 was observed. In addition to the undegraded 1 (recovered only in the feces) 10 metabolites were isolated from the urine and feces as both organic extractable and conjugated products. The metabolic pathways of 1 involved hydroxylation and oxidation at the benzyl and cineole portion of the parent molecule, conjugation (with glucuronic acid and glycine), and ether cleavage (Figure 1).

During the analysis of the urine from animals administered a single dose of 450 mg/kg, two minor organic extractable metabolites (each accounting for less than 1% of the administered dose) were isolated. They were identified as the *O*-acetyl analogues of *o*-(hydroxymethyl)benzoic acid (6) and 9-hydroxy-α-carboxycinmethylin (4). Their identification represented a discovery of a novel conjugation pathway involving the acylation of the hydroxyl moiety. To the best of our knowledge, this is the first reported example of O-acylation of xenobiotics. Due to the potential significance of this finding, this report describes the isolation and identification of these two *O*-acetyl conjugates.

EXPERIMENTAL SECTION

A detailed description on the synthesis of radiolabeled test material and reference standards, treatment of test animals, and the analysis of excreta has been reported (Lee et al., 1986). A brief summary of the experimental design is presented in the following outlines.

Test Compound. The radiochemical purity of [U-*phenyl*-^{14}C]cinmethylin (1) was greater than 99% as determined by two-dimensional thin-layer chromatography (TLC) and liquid scintillation system (LSC). Radioactive impurities greater than 0.5% were not detected.

Test Animals. Male and female rats (Fischer 344, 10–16 weeks of age, weighing from 150 to 200 g each) were obtained from the Simonsen's Laboratories, Gilroy, CA.

Route of Administration. [^{14}C]Cinmethylin was formulated in propylene glycol. An appropriate dose (450 mg/kg) was administered to the test animals by stomach intubation at a constant volume dose of 1.7 mL/kg. The final specific activity of the [^{14}C]cinmethylin treatment solution was 0.16 μCi/mg. Animals were sacrificed 4 days postdosing.

Urine Sampling and Analysis. Urine from the [^{14}C]cinmethylin-treated animals was collected daily. For the qualitative characterization of urinary degradation products, day 1 and day 2 excreta from each animal were combined for analysis. The pH of the combined urine sample was adjusted to pH 3 with 6 N HCl and partitioned three times with equal volumes of ethyl acetate. The organic extract was dried over anhydrous sodium sulfate, concentrated, and analyzed by two-dimensional TLC.

Separation of the organic extractable metabolites was carried out by preparative TLC prior to chromatographic and spectroscopic characterization. Confirmation of structure was obtained by direct comparison with an authentic standard.

Radioassay and Analytical Procedures. Radioactivity was quantitated in 15 mL of Aquasol-2 scintillation solution in a Packard Model 2660 or 300 liquid scintillation system. Extractable radioactivity was analyzed by TLC (silica gel F-254, 0.2 mm, E. Merck). The following solvent combinations were used: (1) toluene–2-propanol–acetic acid (150:20:1.5); (2) hexane–2-propanol–acetic acid (120:30:1); (3) toluene–ethyl acetate (17:3). Radioactivity on the TLC plate was visualized by autoradiography on

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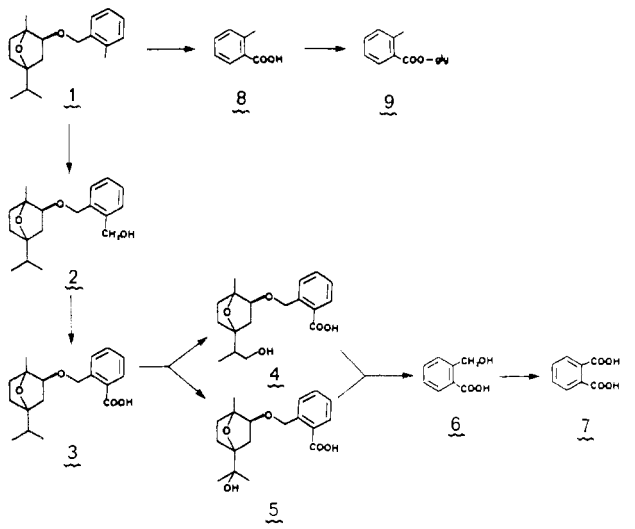


Figure 1. Primary metabolic pathway of [^{14}C]cinmethylin (1) in rats following oral dose administration (Lee et al., 1986).

Table I. Summary of the Chemical Structure of the Cinmethylin Urinary Metabolites (see Figure 2A)

radioactive band	R_f	identified products
A	0.50	o-(hydroxymethyl)benzoic acid lactone
B	0.44, 0.46	α-hydroxycinmethylin (2), α-carboxycinmethylin (3)
C	0.41	8-hydroxy-α-carboxycinmethylin (5)
D	0.37	9-hydroxy-α-carboxycinmethylin (two diastereomers) (4)
E	0.30	o-(hydroxymethyl)benzoic acid (6)
F	0.25	N-(2-methylbenzoyl)glycine (9)

Kodak SB-5 single-coated x-ray film. GC/mass spectroscopy was carried out on a Finnigan 1020 mass spectrometer.

Synthesis of o-(Acetoxymethyl)benzoic Acid (10). o-(Hydroxymethyl)benzoic acid (6) was dissolved in 2 mL of a 1:1 mixture of pyridine–acetic anhydride and kept at room temperature for 2 h. The sample was purified by TLC in solvent system 1. The major product was scraped from the plate and eluted from the silica gel with ethyl acetate. The purified component was analyzed by NMR, IR, and MS. NMR (360 MHz, $CDCl_3$, ppm): 8.14 (1 H, dd), 7.60 (1 H, dt), 7.53 (1 H, d), 7.43 (1 H, dt), 5.59 (2 H, s, CH_2), 2.17 (3 H, s, methyl). IR (CH_2Cl_2, cm^{-1}): 3063, 1738, 1695, 1605, 1580, 1493, 1452, 1410, 1381, 1364, 1300, 1231, 1150, 1036. MS (m/z (%)): underivatized, 151 (32), 134 (58), 133 (100), 123 (10), 105 (76), 89 (51), 79 (10), 77 (47), 51 (17), 45 (11), 43 (53); trimethylsilyl ester, 266 (M^+, 1), 223 (29), 133 (100), 117 (44), 105 (14), 90 (8), 89 (7), 77 (15), 75 (42), 73 (17), 45 (13), 43 (31).

Synthesis of 9-(Acetoxymethyl)-α-carboxycinmethylin (11). 9-Hydroxy-α-carboxycinmethylin (4) (a mixture of two diastereomers; ca. 100 mg) was dissolved in 2 mL of a 1:1 mixture of pyridine–acetic anhydride and the resultant mixture kept at room temperature for 2 h. The sample was purified by TLC in solvent system 1. The major product was scraped from the plate and eluted from the silica gel with ethyl acetate. The purified component (a mixture of two diastereomers) was analyzed by NMR, IR, and MS. NMR (360 MHz, $CDCl_3$, ppm): 8.03 (1 H, aromatic), 7.71 (1 H, aromatic), 7.55 (1 H, aromatic), 7.36 (1 H, aromatic), 4.7–5.0 (2 H, CH_2O), 4.23 (1 H), 3.9–4.15 (2 H, m), 3.67 (1 H, CH, dd), 2.36 (1 H, m), 2.06 (3 H, s, methyl), 1.53 and 1.51 (3 H, s, diastereomeric methyls), 1.08 and 1.06 (3 H, d, diastereomeric isopropyl methyls). IR (CH_2Cl_2, cm^{-1}): 2976, 1732, 1695, 1468, 1368, 1132, 1121, 1103, 1065, 1040. MS (m/z (%)): underivatized, 304 (1), 167 (4), 149 (3), 135 (23), 110 (9), 79 (16), 77 (15), 69 (8), 55 (8), 43 (100); methyl ester, 376 (M^+, <1), 344 (2), 318 (2), 227 (4), 167 (20), 151 (10), 149 (100), 133 (11), 121 (8), 119 (13), 109 (15), 107 (19), 91 (23), 69 (12), 43 (85).

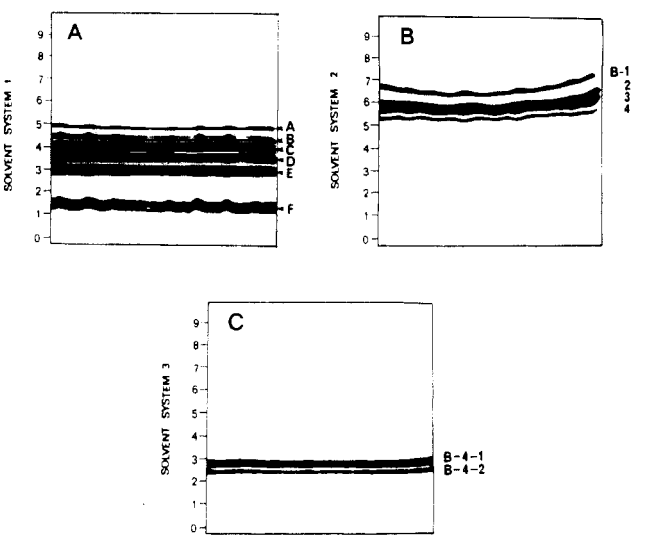
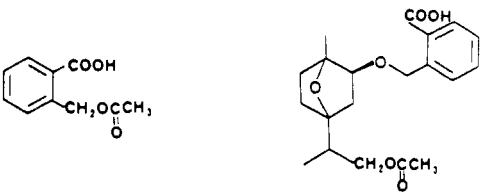


Figure 2. Preparative TLC autoradiograms: (A) organic extractable metabolites from the combined male urine (see Table I for compound designation); (b) preparative separation of radioactive band B; (c) preparative separation of radioactive band B-4.



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RESULTS AND DISCUSSION

Initial preparative TLC separation of the organic extractable urinary metabolites was carried out in solvent system 1. Six major radioactive bands were resolved (Figure 2A). The area of the preparative TLC plate, which corresponded to the designated radioactive band, was removed, and radioactivity was extracted from the silica gel with ethyl acetate as the eluting solvent. The concentrated ethyl acetate extract was subjected to additional preparative TLC, using other solvent systems, for further cleanup and separation prior to spectroscopic analysis. A summary of the various metabolites associated with each band is presented in Table I.

During the second preparative TLC separation of radioactive band B (solvent system 2, Figure 2B), four radioactive bands were resolved. Band B-1 (R_f 0.68) was identified as o-toluic acid (8). Band B-2 (R_f 0.58) and B-3 (R_f 0.60) were identified as α-hydroxycinmethylin (2) and α-carboxycinmethylin (3), respectively. A fourth radioactive band (B-4, R_f 0.53) was also detected. It accounted for approximately 1.5% of the administered radioactivity. Subsequently, a third preparative TLC separation using solvent system 3 was carried out. The autoradiogram presented in Figure 2C revealed that band B-4 actually consisted of two components designated as components B-4-1 (R_f 0.28) and B-4-2 (R_f 0.25). They accounted for approximately 0.8 and 0.4% of the administered radioactivity, respectively.

The mass spectral properties of component B-4-1 are summarized: m/z 151 (M^+ – $CH_3C{=}O$), 149 (M^+ –

COOH), 135 (M^+ – OAc), 133 (m/z 151 – H_2O), 105 (m/z 151 – COOH), 77, 43. This mass spectrum is consistent with the reference standard of *o*-(acetoxymethyl)benzoic acid (**10**).

A trimethylsilyl derivative of component B-4-1 was also analyzed by mass spectroscopy. Characteristic ions were seen at m/z 266 (M^+), 251 ($M^+ - CH_3$), 223 ($M^+ - CH_3C{=}O$), 207 (M^+ – OAc), 133 (m/z 223 – Me_3SiOH), 117 ($COOMe_3Si$), 105, 90, 89, 75, and 73. This spectrum was consistent with the trimethylsilyl derivative of **10**. The retention times of the metabolite and standard were also nearly identical. Component B-4-1 is confirmed as *o*-(acetoxymethyl)benzoic acid.

Component B-4-2 was methylated prior to GC/MS analysis. This radioactive component eluted at approximately 250 °C. An expanded chromatogram showed that it actually consisted of two closely related components. The mass spectra of these two components were identical and showed the presence of a molecular ion (M^+) at m/z 376 and other fragment ions at m/z 344, 318, 227, 167, 149, 133, 119, and 43. These mass spectra are consistent with the mass spectrum of the methyl ester of 9-(acetoxymethyl)-α-carboxycinmethylin (**11**). Component B-4-2 was also analyzed by GC/MS as the trimethylsilyl derivative. Both the retention time and the mass spectrum of the metabolite matched those of the standard.

It is evident that 9-(acetoxymethyl)-α-carboxycinmethylin (**11**) was generated as the *O*-acetyl conjugate of 9-hydroxy-α-carboxycinmethylin (**4**) (also isolated as two diastereomers). **10** and **11** were recovered as unique metabolites of cinmethylin (**1**) since they were not detected during the isolation and identification of their corresponding precursors, *o*-(hydroxymethyl)benzoic acid (**6**) and 9-hydroxy-α-carboxycinmethylin (**4**), respectively, under identical conditions. Similar results were observed with methanol as the extraction solvent of the silica gel.

CONCLUSION

Conjugations are biosynthetic reactions in which xenobiotics or their metabolites react with readily available, endogenous substances such as glucuronic acid, sulfate, acetate, amino acid, etc. These endogenous substrates are transferred from the coenzymes participating in intermediary metabolism to usual conjugation reaction sites such as the hydroxyl, carboxyl, epoxide, halogen, thiono, and amino functional groups. The best understood acylation reaction to date is limited to the transfer of acetate from acetyl coenzyme A to an amino group [see examples provided by Iwan (1976)]. Several examples of the N-acylation reaction of pesticide molecules are known and include metobromuron (Tweedy et al., 1970) and 4,6-dinitro-*o*-cresol (Smith et al., 1953). O-Acylation of xenobiotics has not yet been reported. In the rat metabolism study of cinmethylin, O-acylation products of *o*-(hydroxymethyl)benzoic acid and 9-hydroxy-α-carboxycinmethylin, principal metabolites of cinmethylin, were isolated. The formation of these novel conjugates is of biochemical significance since these conjugates have a higher lipid solubility than the free molecule.

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Registry No. **1**, 87818-31-3; **2**, 99765-53-4; **3**, 87819-14-5; **4**, 110901-97-8; **5**, 110826-67-0; **6**, 612-20-4; **6** (lactone), 87-41-2; **9**, 42013-20-7; **10**, 35461-75-7; **11**, 110826-66-9.

LITERATURE CITED

Iwan, J. "Miscellaneous Conjugates—Acylation and Alkylation of Xenobiotics in Physiologically Active Systems". In *Bound and Conjugated Residue Residues*; Kaufman, D. D., Still, G. G., Paulson, G. D., Bandal, S. K., Eds.; American Chemical Society: Washington, DC, 1976; pp 132–152.

Lee, P. W.; Stearns, S. M.; Powell, W. R.; Stoutamire, D. W.; Payne, G. B.; Woodward, M. D.; Burton, W. B.; Silveira, E. J.; Ehmann, A. "Metabolic Fate of Cinmethylin in Rats". *J. Agric. Food Chem.* **1986**, *34*, 162.

Smith, J. N.; Smithies, R. H.; Williams, R. T. "Studies in Detoxication 48. Urinary Metabolites of 4,6-Dinitro-*o*-cresol in the Rabbit". *Biochem. J.* **1953**, *54*, 225.

Tweedy, B. G.; Loeppky, C.; Ross, J. A. "Metabolism of 3-(*p*-Bromophenyl)-1-methoxy-1-methylurea (Metobromuron) by Selected Soil Microorganisms". *J. Agric. Food Chem.* **1970**, *18*, 851.

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Assessment of Folate Bioavailability in the Rat Using Extrinsic Dietary Enrichment with Radiolabeled Folates

Ana R. Abad and Jesse F. Gregory III*

The relative bioavailability of tritiated folates was examined in the rat by a single-dose protocol. Hepatic [^{3}H]folate retention 24 h postdose and 24-h urinary tritium excretion were used as primary response criteria. The in vivo retention of the tracer dose of [^{3}H]folic acid (0.11 nmol) was not significantly affected by the level of total dietary folate over the range of 0–10 nmol of added folic acid but was reduced by the presence of 100 nmol of folic acid. Monoglutamyl [^{3}H]tetrahydrofolates (mainly 5-formyl) from bacterial synthesis exhibited greater in vivo retention than either their polyglutamyl analogues or [^{3}H]folic acid monoglutamate. In additional studies, the presence of cabbage and orange juice in test meals significantly retarded the bioavailability of [^{3}H]folic acid and bacterial polyglutamyl [^{3}H]folates, while pectin, wheat bran, and kidney beans exhibited trends toward reduced bioavailability of the labeled folates.

The folate nutriture of humans and animals depends on the content and bioavailability of the dietary folates relative to the nutritional requirement for the vitamin. A great deal of uncertainty exists concerning the bioavailability of folate. In the context of this study, we define bioavailability as the overall utilization of the vitamin including intestinal absorption and function in folate

Food Science and Human Nutrition Department, University of Florida, Gainesville, Florida 32611.

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