Palo Alto, Calif.
(10) S. J. Mule and P. L. Hushin, *Anal. Chem.*, **43**, 708 (1971).
(11) D. C. Monkhouse and J. L. Lach, *J. Pharm. Sci.*, **61**, 1435 (1972).
(12) K. Oguri, S. Ida, H. Yoshimura, and H. Tsukamoto, *Chem. Pharm. Bull.*, **18**, 2414 (1970).
(13) J. Cochin, J. Haggart, L. A. Woods, and M. H. Seevers, *J. Pharmacol. Exp. Ther.*, **111**, 74 (1954).
(14) S. Y. Yeh, H. I. Chernov, and L. A. Woods, *J. Pharm. Sci.*, **60**, 469 (1971).

Identification of Degradation Products in a Phenylbutazone Tablet Formulation

F. MATSUI ˣ, D. L. ROBERTSON, M. A. POIRIER, and E. G. LOVERING

Received April 27, 1979, from the *Drug Research Laboratories, Health Protection Branch, Tunney's Pasture, Ottawa, Ontario, Canada, K1A 0L2.* Accepted for publication October 24, 1979.

Abstract □ Two previously reported but unidentified phenylbutazone degradation products were isolated from a tablet that was stored at 60° for 203 days. The compounds, α-(*N*-phenylcarbamoyl)-*N*-caproylhydrazobenzene and α-hydroxy-α-(*N*-phenylcarbamoyl)-*N*-caproylhydrazobenzene, were isolated by chromatography, identified by mass and NMR spectrometry, and synthesized by the reaction of aniline with phenylbutazone or its hydroxy analog, respectively.

Keyphrases □ Phenylbutazone—tablets, identification of degradation products □ Anti-inflammatory agents—phenylbutazone, tablets, identification of degradation products □ Degradation products—phenylbutazone, tablets, identification

In a recent study on the stability of phenylbutazone formulations under stress conditions (1), one formulation (G) was found to contain one or more compounds previously unidentified in degradation of the drug. These compounds are α-(*N*-phenylcarbamoyl)-*N*-caproylhydrazobenzene (I) and α-hydroxy-α-(*N*-phenylcarbamoyl)-*N*-caproylhydrazobenzene (II). This paper describes the isolation, identification, and synthesis of I and II.

EXPERIMENTAL

Materials—Tablets of Formulation G (1) were obtained directly from the manufacturer. Phenylbutazone[1] (*III*), cyclohexane[2], chloroform[2], methanol[2], ethyl acetate[2], and acetic acid[3] were used as received. Phenyl phthalate[4] was recrystallized from acetone[2]–water, and 1,2-diphenyl-4-*n*-butyl-4-hydroxypyrazolidine-3,5-dione (IV) was prepared by the method described previously (1, 2). TLC plates[5] precoated with silica gel G-60 F-254, 60–200-mesh silica gel[6], diatomaceous earth[7], and nylon tubing[8] were used.

Equipment—The gas chromatograph[9] was equipped with a flame-ionization detector and a U-shaped glass column packed with 5% OV-7 on Gas Chrom Q[7] (100–120 mesh). NMR spectra were obtained on a 90-MHz instrument[10]; chemical shifts are reported in parts per million from tetramethylsilane, the internal standard. All spectra were recorded in deuterated chloroform[11] ($CDCl_3$). Mass spectra[12] were recorded at an ionizing potential of 70 ev, and the samples were introduced *via* the direct probe at 120 and 130°. Accurate mass measurements were made by the peak matching technique.

TLC Solvent System A (3) consisted of cyclohexane–chloroform–methanol–acetic acid (60:30:5:5), and Solvent System B was chloroform–ethyl acetate (80:20). Filter paper-lined TLC tanks were equilibrated with the solvent system for 30 min prior to use. Spots were visualized by UV light at 254 nm and by spraying with a solution of 0.5% potassium dichromate in 20% (v/v) H_2SO_4.

Isolation of Degradation Products from Formulation G—Five tablets (equivalent to 500 mg of phenylbutazone), which had been stored for 203 days at 60°, were finely powdered, wetted with ethyl acetate, and triturated with 2 g of diatomaceous earth until the mixture was uniform. The triturate was placed at the top of a nylon column containing 200 g of 60–200-mesh silica gel, which had been deactivated with 30 ml of water and dry packed into a 0.03 × 1-m nylon column. A 500-ml solution of Solvent System A was added until the solvent front reached the bottom of the column. The tube was incised into 5-cm sections, and the location of each compound on the column was established by removing small portions of the silica gel from the different incised sections of the nylon tube, extracting these portions with ethyl acetate, and spotting aliquots directly onto a TLC plate.

The spots were visualized with the potassium dichromate spray, and the spots containing the components of interest immediately developed a bright-purple color. The sections of interest then were cut; the degradation products were extracted from the silica gel with ethyl acetate, concentrated under nitrogen, and rechromatographed on a TLC plate using Solvent System A to remove residual phenylbutazone. The R_f values of I, II, and III in this system were 0.37, 0.37, and 0.43, respectively.

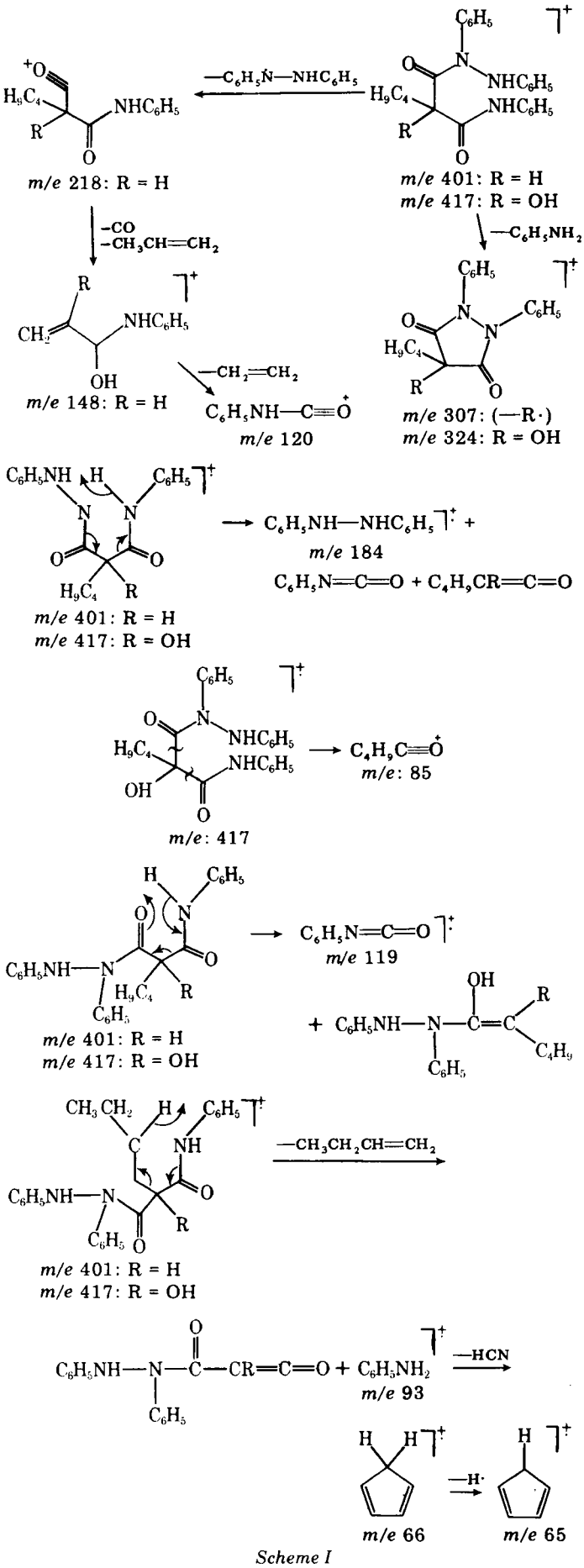
The band containing the mixture of I and II was visualized by spraying the edges of the TLC plate with dichromate spray. The band was scraped off and extracted with ethyl acetate, and the eluate was reduced to a small volume under nitrogen. Resolution of I and II was effected by TLC using Solvent System B. Isolation was carried out by elution with ethyl acetate and evaporation under nitrogen. The R_f values of I, II, and III using Solvent System B were 0.56, 0.50, and 0.58, respectively.

Syntheses—*Compound I*—A solution of 308 mg (1 mmole) of III and 180 mg of aniline (100% excess) in 15 ml of chloroform was heated under nitrogen at 45° for 48 hr in a culture tube. Compound I was separated from unreacted phenylbutazone by TLC (System A), and the spot was scraped from the plate and eluted with ethyl acetate. Traces of II were removed by subsequent TLC using Solvent System B. Crystallization from ethanol–water (90:10) gave pure I, mp 197–199°; NMR ($CDCl_3$): δ 8.9 (s, NH), 8.5 (s, CONH), 7.4–6.8 (m, 15H, aromatic), 4.2 (t, CH), 2.0, 1.5, and 0.87 (*n*-butyl) ppm; mass spectrum (70 ev, 120°): *m/e* 401 (M^+ 5.3%), 307 (7.1), 218 (12.5), 184 (48.1), 148 (13.1), 120 (18.1), 93 (100), and 77 (72.3). The GLC[13] retention time was 11.0 min or 0.92 relative to the internal standard, phenyl phthalate.

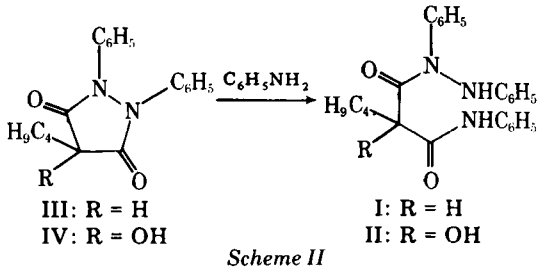
Compound II—Compound II was prepared from 1,2-diphenyl-4-*n*-butyl-4-hydroxypyrazoline-3,5-dione (IV) by the procedure described for the synthesis of I. Compound II also can be obtained from III by carrying out the reaction under an oxygen atmosphere. After purification by TLC, crystallization from ethanol–water (90:10) yielded II, mp

[1] Mount Royal Chemicals, Montreal, Quebec, Canada.
[2] Caledon Laboratories, Georgetown, Ontario, Canada.
[3] J. T. Baker Chemical Co., Phillipsburg, N.J.
[4] Aldrich Chemical Co., Milwaukee, Wis.
[5] Brinkmann Instruments, Toronto, Ontario, Canada.
[6] E. Merck, Darmstadt, West Germany.
[7] Celite 545, Chromatographic Specialties, Brockville, Ontario, Canada.
[8] ICN Pharmaceuticals, Cleveland, Ohio.
[9] Varian Aerograph 2100.
[10] Bruker HFX-90 spectrometer.
[11] Stohler isotope chemicals, Waltham, Mass.
[12] AEI MS-9 mass spectrometer.
[13] With 5% OV-7 on Gas Chrom Q (100–120 mesh) in a U-shaped glass column under isothermal conditions at 230°. All other conditions were described previously (4).

0022-3549/80/0400-0469$01.00/0



Scheme I



Scheme II

175–176°; NMR ($CDCl_3$): δ 9.4 (s, NH), 9.0 (s, CONH), 7.5–7.2 (m, 15H, aromatic), 5.4 (broad, OH), 2.0, 1.2, and 0.70 (*n*-butyl) ppm; mass spectrum (70 ev, 130°): *m/e* 417 (M^+ 10.3%), 324 (11.5), 207 (12.7), 184 (100), 164 (16.5), 119 (13.4), 93 (89.5), 85 (28.3), 81 (14.2), and 77 (77.3). The GLC[13] retention time was 11.0 min or 0.92 relative to the internal standard, phenyl phthalate.

RESULTS AND DISCUSSION

The mass spectra of I and II contained major peaks at *m/e* 401 (5.3%) and 417 (10.3), respectively, corresponding to their molecular ions. The possible fragmentation patterns of I and II are presented in Scheme I. The mass spectra of I and II differ from those of their respective precursors, III and IV (2, 5), in that they exhibit major losses of aniline at *m/e* 93 (100 and 90%, respectively), indicating the presence of open-ring compounds. The loss of aniline seems to occur with ring closure in the mass spectrometer, giving ions at *m/e* 307 (7.1%) and 324 (11.5). Most fragments in the mass spectrum can be rationalized by McLafferty rearrangements (5, 6).

The most common ions in the mass spectra of pyrazolidinediones are the series of peaks at 183, 184, and 185, which are characteristic of hydrazobenzene fragments. The ion at *m/e* 184 may originate from the molecular ions of I or II by a McLafferty rearrangement with a loss of phenyl isocyanate. A characteristic fragment in pyrazolidinediones is the loss of phenyl isocyanate as a neutral molecule or as an ion at *m/e* 119 (5.1% for I and 13.4% for II). The formation of the fragment at *m/e* 184 in the mass spectra of I and II is rationalized by a proton transfer from the anilide portion (2). The formation of the ion at *m/e* 85 in II, attributable to (C_4H_9–C≡O$^+$), indicates the presence of the hydroxyl group at carbon 4.

The NMR spectra of I and II in deuterochloroform showed two singlets due to NH protons at δ 8.9 and 8.5 and 9.4 and 9.0 ppm, respectively. One of these NH signals corresponds to an amide function. This assignment is supported by the chemical shifts and by the fact that one singlet disappears slowly when deuterated water is added, while the other disappears immediately. The chemical shifts of the methine proton in I (4.2 ppm, triplet, J = 8.0 Hz, HCCH) and 5.4 ppm for the hydroxylic proton in II are in agreement with the chemical shifts of the equivalent proton in the free α-carboxy compounds reported previously (7). Integration of the aromatic protons revealed the presence of three aromatic rings. The complexity of the aromatic pattern in the spectra of I and II, as compared to that of III, suggests that the plane of symmetry was destroyed, an observation that supports the proposed structure of I and II.

Compounds I and II may have formed in Formulation G by the reaction of aniline with III and IV, respectively (Scheme II). The source of the aniline required for the formation of these compounds is not known. It may originate from the degradation of III by an unusual excipient-induced mechanism. The presence of II in the tablets can be rationalized in terms of air oxidation of I at carbon 4 (2). On the basis of previous results (1), the ring opening of the lactam-type compounds by aniline seems to be more probable than hydrolysis of III and IV to α-carboxy-*N*-caproylhydrazobenzene and α-carboxy-α-hydroxy-*N*-caproylhydrazobenzene, respectively (2, 7), followed by combination with aniline. Antacid formulations would be expected to show degradation *via* hydrolysis, and the presence of relatively high concentrations of carboxy compounds in antacid formulations subjected to 60° storage for 18 months is in accord with this expectation. These antacid formulations showed only traces of the previously unidentified I and II, thus giving indirect evidence for the ring-opening reaction with aniline.

REFERENCES

(1) F. Matsui, D. L. Robertson, P. Lafontaine, H. Kolasinski, and E. G. Lovering, *J. Pharm. Sci.*, **67**, 646 (1978).

(2) D. V. C. Awang and A. Vincent, *ibid.*, **65**, 68 (1976).

(3) H. D. Beckstead, K. K. Kaistha, and S. J. Smith, *ibid.*, **57**, 1952 (1968).
(4) J. R. Watson, F. Matsui, R. C. Lawrence, and P. M. J. McConnell, *J. Chromatogr.*, **76**, 141 (1973).
(5) R. A. Locock, R. E. Moskalyk, L. G. Chatten, and L. M. Lundy, *J. Pharm. Sci.*, **63**, 1896 (1974).
(6) B. Unterhalt, *Arch. Pharm.*, **305**, 334 (1972).
(7) G. A. Neville and D. V. C. Awang, *Org. Mag. Res.*, **2**, 341 (1970).

Stability-Indicating Colorimetric Assay for Indicine *N*-Oxide Using TLC

JOSEPH B. D'SILVA and ROBERT E. NOTARI ˣ

Received May 18, 1979, from the *College of Pharmacy, Ohio State University, Columbus, OH 43210.* Accepted for publication October 31, 1979.

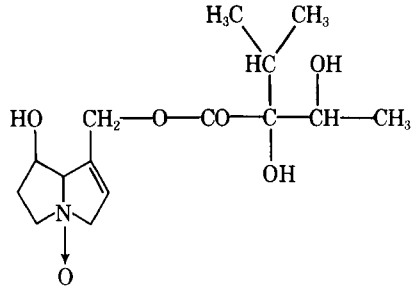
Abstract □ Aliquots of aqueous solutions in which indicine *N*-oxide may be degraded were mixed with 0.5 *M* formic acid (1:3) to adjust the pH to ~2–4 to quench the reaction and to ensure adequate TLC resolution. Silica-coated aluminum sheets were used to isolate indicine *N*-oxide by cutting the appropriate region from the chromatogram. By a modification of a known procedure, the silica gel then was treated with an acetic anhydride–diglyme mixture, and the mixture was heated to convert the drug to a pyrrole, which was then coupled with 4-dimethylaminobenzaldehyde to produce a color. The absorbance of the resulting solution was determined at 566 nm, and the apparent molar absorptivity, ϵ, based on the final indicine *N*-oxide concentration was 6.13×10^4. The recovery was ~92%, and the assays were readily reproducible with a coefficient of variation of 4.4%.

Keyphrases □ Indicine *N*-oxide—stability-indicating colorimetric assay using TLC □ Alkaloids—indicine *N*-oxide, stability-indicating colorimetric assay using TLC □ Antineoplastic agents, potential—indicine *N*-oxide, stability-indicating colorimetric assay using TLC

Indicine *N*-oxide[1] (I), an unsaturated pyrrolizidine alkaloid found in *Heliotropium indicum* Linn (Boraginaceae) (1), is undergoing clinical testing as an anticancer agent (2). While stability data have not been reported, alkaline ester hydrolysis is predicted from degradation studies of related alkaloids.

BACKGROUND

Approximate half-lives for the decomposition of 12 pyrrolizidines were estimated in 0.5 *N* aqueous or hydroalcoholic sodium hydroxide at room temperature (3). The relatively facile hydrolysis of esters of trachelanthic and viridifloric acids was attributed to their potential for β-hydroxyl participation, presumably *via* hydrogen bonding (3). This potential for intramolecular catalysis is present in I, which also is a trachelanthic acid ester (of retronecine *N*-oxide). The products obtained from the hydrolysis of indicine in 2 *N* NaOH at 100° for 2 hr were shown to be retronecine and a diastereoisomer of trachelanthic acid (4). It is not known whether the presence of the *N*-oxide in I gives rise to additional degradation pathways. Kugelman *et al.* (1) found that the properties of I extracted from *H. indicum* Linn (Boraginaceae) did not agree with those of synthesized I. Although these differences were ascribed to solvation problems, they also might reflect chemical instability.



I

Two assays for I in biological samples have been reported (2, 5). An electron-capture GLC assay after formation of the pentafluoropropionic anhydride derivative of indicine was applied to the analysis of mixtures of indicine and I in plasma and urine (2). Prior to analysis, indicine was extracted selectively with chloroform; the I remaining in the raffinate then was reduced to indicine. A GLC–mass spectrometric method using selective-ion monitoring recently achieved nanogram sensitivity through formation of the trimethylsilyl derivative of I (5). Although the method is selective for I, the equipment required is sophisticated and expensive.

This study was undertaken to develop a simple, specific assay for I in the presence of its degradation products. A colorimetric assay for unsaturated pyrrolizidine alkaloids using modified Ehrlich reagent (6) was adapted to assay I under aqueous conditions, in which it was shown to be unstable. TLC on aluminum sheets was employed to isolate I from buffers and reaction products. After the appropriate region was cut and scraped, the silica gel mixture was treated to convert the pyrrolizidine structure to a pyrrole. The pyrrole then was coupled with 4-dimethylaminobenzaldehyde to produce a color which was measured spectrophotometrically.

EXPERIMENTAL

Materials and Chemicals—The TLC aluminum sheets were precoated with 0.2 mm of silica gel 60 F-254[2]. Ether[3], absolute ethanol[4], ammonium hydroxide solution[5], acetic anhydride[6], and acetone[3] were analytical reagent grade. The diglyme[7] was kept free of peroxides (6). Modified Ehrlich reagent was prepared by dissolving 2% (w/v) 4-dimethylaminobenzaldehyde[8] in an ethanolic solution containing 14% (w/v) boron trifluoride, which was incorporated as its etherate complex (6, 7).

pH Adjustment—The assay results, obtained from absorbance values, were consistent provided that the sample to be spotted had a pH of 2–4.5. At pH < 2, erratic absorbance values were obtained. The pH was maintained at <4.5 since I degraded in alkali but not in acid. One part of 0.5 *M* formic acid was effective in controlling the pH when it was mixed with three parts of simulated reaction solutions of pH 2–13. Indicine *N*-oxide solutions treated in this manner were stable for several weeks under refrigeration.

Optimum Conditions—The amount of acetic anhydride used in the conversion of I to the pyrrole was varied from 0.1 to 0.4 ml. The best results were obtained with 0.2 ml. The optimum heating time for this step was 3.5 min. An optimum heating period of 4.5 min was observed for the color-producing step using modified Ehrlich reagent.

Assay of I in Aqueous Solutions or Reaction Mixtures—An aliquot of ~0.01 *M* I solution or reaction mixture at pH 2–13 was mixed with

[1] NSC-132319.

[2] EM Laboratories, Elmsford, N.Y.
[3] Mallinckrodt, St. Louis, Mo.
[4] U.S. Industrial Chemicals Co., New York, N.Y.
[5] Allied Chemical Co., Morristown, N.J.
[6] Drake Brothers, Menomonee Falls, Wis.
[7] Aldrich Chemical Co., Milwaukee, Wis.
[8] J. T. Baker Chemical Co., Phillipsburg, N.J.

0022-3549/80/0400-0471$01.00/0