Microbial Transformations of N-Methylcolchicineamide

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Seventy-seven microorganisms were examined for their ability to metabolize the antineoplastic agent N-methylcolchicineamide, an analog of colchicine. Five streptomycetes exhibited significant metabolism, and Streptomyces griseus NRRL B-599 completely converted the substrate to three metabolites. In preparative-scale studies, N-dealkylation resulted in the production of colchicineamide, the major metabolite (65%), which was characterized by chemical, spectroscopic, and chromatographic comparisons with the standard compound. Two phenolic metabolites resulting from O-dealkylation were also isolated and identified as 2- and 3-O-demethyl-N-methylcolchicineamide.

N-Methylcolchicineamide (Fig. 1, compound I) represents the vinylogous amide-analog of the vinylogous ester, colchicine (Fig. 1, compound II) (1). Although the major medicinal use of colchicine is in the relief of acute attacks of gouty arthritis, recent interest in colchicine derivatives stems from their potential use as antineoplastic agents (13). The narrow therapeutic index of the parent compound (II) limits its usefulness in this regard, but analogs such as I hold more promise due to increased potency, decreased toxicity, or both (11, 14).

Several reports have appeared concerning the microbial metabolism of colchicine (II). Velluz and Bellet (17; Roussel-UCLAF, British patent 923,421, Chem. Abstr. 59:13320a, 1963) utilized Streptomyces griseus ATCC 10137 to O-dealkylate colchicine, although the product was not fully characterized. The same organism was found to S-dealkylate thiocolchicine (Fig. 1, compound III) to yield compound IV (Fig. 1). More recently, Hufford et al. (7) described the O-dealkylation of colchicine in the 2- and 3-positions by Streptomyces spectabilis ATCC 27465 or S. griseus ATCC 13968. The side-chain degradation of colchicine by Arthrobacter colchovorum, as reported by Zeitler and Niemer (19), included decacylation in the absence of a carbon source and oxidative cleavage of the entire acetamide side chain in the absence of a nitrogen source. The microbial metabolism of other colchicine derivatives has not been described.

This report describes the quantitative metabolism of N-methylcolchicineamide by S. griseus NRRL B-599 to yield colchicineamide (Fig. 1, compound V) and minor amounts of the phenols VI and VII (Fig. 1). These studies were undertaken with the intention of applying microbial systems to the preparation of metabolites for biological evaluation, and to suggest parallels in the metabolism of this antitumor agent in regard to potential bioactivation or detoxification that may occur in mammalian systems (12).

MATERIALS AND METHODS

Materials. All solvents were analytical reagent or better in quality. Solvents for high-performance liquid chromatography were of HPLC grade (Lichrosolv; MCB Manufacturing Chemists, Cincinnati, Ohio). Diazald and a diazomethane-generating apparatus were purchased from the Aldrich Chemical Co.

Analytical methods. Proton nuclear magnetic resonance (NMR) spectra were generated in either CDCl₃ or dimethyl sulfoxide-D₆ using tetramethysilane as the internal standard on a Varian HA-100 (100 MHz) spectrometer. Mass spectra were taken on a Du Pont model 21491 mass spectrometer by direct probe insertion. Melting points were obtained with a Fisher model 355 digital melting point apparatus and are corrected.

Analytical thin-layer chromatography (TLC) was performed on plastic-backed 0.25-mm Silica Gel GF₂₅₄ plates (Polygram, Brinkman) eluted with one of the following solvent systems: A, chloroform-acetone-diethylamine (7:3:1); B, benzene-ethyl acetate-diethylamine-methanol (50:40:10:8); R₃ values for compounds are reported. Solvent systems B: N-methylcolchicineamide (I), 0.51; colchicineamide (V), 0.40; VI, 0.13; and VII, 0.07. R₃ values in system B were: N-methylcolchicineamide, 0.34; colchicineamide, 0.15; VI, 0.11, and VII, 0.05. Plates were visualized by quenching under 254-nm light or fluorescence under 280-nm light, followed by air oxidation overnight. Preparative TLC was conducted on 1-mm Silica Gel PF₂₅₄₄ plates, 20 by 20 cm (Brinkman), eluted with solvent system A. High-performance liquid chromatography was conducted as described earlier (3) on a Bondapak C-18 column (Waters Associates, Milford, Mass.) eluted with phosphate buffer (pH 6.0, 0.022 M, μ = 0.05)—acetoniitrite-methanol (79:16:5) at a flow rate of 2 ml/min. Under these conditions retention times were as follows: colchicineamide, 24 min; VI, 10 min; VII, 8 min.

Standard compounds. N-Methylcolchicineamide
was purchased from Aldrich Chemical Co. (Bader Rare Chemical Collection, Milwaukie, Wis.) and gave the following analytical data: mp 229-230°C (reported [5] 230-232°C); NMR (CDCl₃) ppm (multiplicity, assignment) 1.97 (s, 3H, COCH₃), 2.0-2.6 (m, 5H aliphatics), 2.12 (d, J = 6, 3H, N-CH₃), 2.52 (s, 3H, Cl-CH₃), 3.92, 3.96 (2s, 6H, C₂- and C₃-CH₂), 4.74 (br. s, 1H, NHCH₃), 5.66 (s, 1H, H-4), 6.82 (d, J = 11, 1H, H-11), 7.51 (d, J = 11, 1H, H-12), 7.64 (s, 1H, H-8), 8.80 (s, 1H, NHCO), identical to the reported spectrum (4); mass spectrum, m/e (percent relative abundance), 385 (28), 384 (100), 356 (18), 341 (14), 325 (13), 313 (13), and 297 (12), identical to the reported spectrum (18).

Colchicineamide was obtained from J. R. Mead (National Cancer Institute, Bethesda, Md.) and gave the following analytical data: mp 262.4°C (reported [5] 261-262°C); NMR (CDCl₃) ppm (multiplicity, assignment) 1.88 (s, 3H, COCH₃) 2.2-2.6 (m, 5H aliphatics), 3.64 (s, 3H, Cl-CH₃), 3.91, 3.96 (2s, 6H, C₂- and C₃-CH₂), 6.11 (s, 2H, NH₂), 6.54 (s, 1H, H-4), 6.90 (d, J = 11, 1H, H-11), 7.35 (d, J = 11, 1H, H-12), 7.60 (s, 1H, H-8), 8.10 (s, 1H, NHCO), identical to the reported spectrum (4); mass spectrum, m/e (percent relative abundance), 385 (28), 384 (100), 356 (18), 341 (14), 325 (13), 313 (13), and 297 (12), identical to the reported spectrum (18).

Screening and general fermentation conditions. All cultures were maintained in stock culture on refrigerated (2°C) slants composed of either Sabouraud-maltose agar (Difco), Mycophil agar (BBL Microbiology Systems), or ATCC medium 5 sporulation agar. Cultures were transferred to fresh slants every 6 months to maintain viability. The medium used in all studies was a soybean-dextrose medium of the following composition: dextrose, 20 g; soybean meal (20 mesh, Capital Feeds, Austin, Tex.), 5 g; sodium chloride, 5 g; potassium phosphate (dibasic), 5 g; yeast extract (Difco), 5 g; distilled water, 1,000 ml; pH adjusted to 7.0 with 5N HCl. The medium was sterilized in individual flasks at 121°C for 15 min.

Incubations were conducted in 125-ml Belco De-Long culture flasks containing 25 ml of the medium, using an NBS model G-25 Environmental Shaker (New Brunswick Scientific, Edison, N.J.) at 250 rpm and 27°C. For each of 77 cultures, first-stage flasks were initiated by suspending spores from slants in sterile medium and transferring the suspension to a 125-ml flask under aseptic conditions. After incubation for 72 h, a 2-ml portion was used to inoculate a second-stage flask of the same composition for each organism, and the incubation was allowed to continue for 24 h. The substrate, N-methylcolchicineamide, was added at a level of 6.25 μl in 25 μl of dimethylformamide per flask. At daily intervals for 5 days, 2-ml samples were aseptically withdrawn and frozen before analysis.

Samples were thawed, made alkaline with 10 drops of saturated bicarbonate solution, and extracted with 2-ml portions of chloroform. After centrifugation, the organic layer was evaporated to dryness under a nitrogen stream and redissolved in 50 μl of chloroform, and 5-μl samples were spotted on TLC plates for elution with solvent systems A and B. Control incubations were conducted with N-methylcolchicineamide in sterilized medium and in autoclaved cultures which had been shown to possess metabolic capability with the substrate.

Preparative-scale incubation of N-methylcolchicineamide with S. griseus NRRL B-599. Three 125-ml first-stage flasks containing 25 ml of medium were inoculated with S. griseus (NRRL B-599) and incubated under the general fermentation conditions for 72 h. The fully grown cultures were used as inoculum for four 1-liter second-stage flasks, each containing 200 ml of medium. After incubation for an additional 24 h, the substrate (N-Methylcolchicineamide, 50 mg in 200 μl of dimethylformamide) was added to each flask, and the incubation was allowed to continue. Periodic extraction and TLC analysis in systems A and B indicated that the substrate was completely consumed after 3 days.

The combined cultures (800 ml) were adjusted to pH 8.5 with saturated bicarbonate and extracted with chloroform (3 × 500 ml), and the chloroform layer was taken to dryness in vacuo to yield 230 mg of a light brown oil. Partitioning between 30 ml of chloroform and 3 × 60 ml of 10% potassium hydroxide yielded the major metabolite, colchicineamide, in the organic phase, which was further purified by preparative TLC.
using solvent system A to yield 130 mg (65%) of colchicine amide. Crystallization from ethanol yielded a yellow crystalline material which gave the following analytical data: mp 261.3°C (standard colchicine amide mp, 262.4°C); no depression on admixture with the standard (261.2°C) (reported [5] 261-262°C); NMR (CDCl3) ppm (multiplicity, assignment) 1.98 (s, 3H, COCH3), 2.0-2.6 (m, 5H aliphatics), 3.62 (s, 3H, Cl-OCH3), 3.89, 3.93 (2H, CH2- and C3-OCH3), 6.05 (s, 2H, NHs), 6.62 (s, 1H, H-4), 6.89 (d, J = 11, 1H, H-11), 7.34 (d, J = 11, 1H, H-12), 7.56 (s, 1H, H-8), 7.94 (s, 1H, NHCO); identical to the reported spectrum (4) and standard colchicine amide; mass spectrum, m/e (percent relative abundance), 385 (48), 384 (100), 356 (23), 341 (18), 325 (20), 313 (18), and 297 (17), identical with the reported spectrum (18) and standard colchicine amide; the metabolite co-chromatographed with standard colchicine amide on TLC employing solvent systems A and B and on the high-performance liquid chromatography system described. This metabolite was not reconverted to N-methylcolchicine amide by methylation with diazomethane (Diazald, Aldrich) while monitoring in solvent systems A and B.

The aqueous-base fraction from above was neutralized with 6 N HCl, saturated with sodium chloride, and exhaustively extracted with chloroform. The organic layer was dried with anhydrous sodium sulfate, taken to dryness in vacuo, and fractionated by preparative TLC using multiple development in solvent system A to yield an upper band (VI) and a minor lower band (VII). The bands were scraped from the plates, and the metabolites were eluted from the silica with ethanol and taken to dryness in vacuo. Neither phenol could be induced to crystallize. Each gave a similar mass spectrum, M+ = m/e 384. Both compounds were reconverted to N-methylcolchicine amide by methylation with diazomethane (Diazald, Aldrich) while monitoring in solvent systems A and B. The upper metabolite (16 mg) gave the following NMR spectrum: (CDCl3) ppm (multiplicity, assignment) 1.96 (s, 3H, COCH3), 2.1-2.6 (m, 5H aliphatics), 3.12 (d, J = 6, 3H, N-CH3), 3.63 (s, 3H, Cl-OCH3), 3.92 (s, 3H, C3-OCH3), 4.74 (broad s, 1H, NH-CH3), 5.90 (broad s, 1H, C2-CH2), 6.54 (s, 1H, H-4), 6.60 (d, J = 11, 1H, H-11), 7.50 (d, J = 11, 1H, H-12), 7.63 (s, 1H, H-8), 8.61 (s, 1H, NHCO). In dimethyl sulfoxide-D6 the following partial spectrum was obtained: 6.56 (d, J = 11, 1H, H-11), 6.66 (s, 1H, H-4), 7.11 (s, 1H, H-8), 7.24 (d, J = 11, 1H, H-12); addition of 1.5 eq of NaOH (9) gave the following shifted spectrum: 6.37 (s, 1H, H-4), 6.52 (d, J = 11, 1H, H-11), 7.13 (s, 1H, H-8), 7.32 (d, J = 11, 1H, H-12).

The lower metabolite (5 mg) yielded a marginal NMR spectrum with the following discernible assignments: (CDCl3) ppm (multiplicity, assignment) 2.00 (s, COCH3), 2.2-2.6 (m, aliphatics), 3.10 (d, J = 6, N-CH3), 3.62 (s, Cl-OCH3), 4.02 (s, C2-OCH3).

RESULTS AND DISCUSSION

The screening of the 77 cultures was undertaken to discern cultures capable of metabolizing N-methylcolchicine amide. These cultures were selected based on literature reports and observations from our laboratories which indicate that they are capable of oxidative-type reactions on a variety of substrates, including N-dealkylation, O-dealkylation, and aromatic hydroxylation. A similar consistent pattern of metabolism was observed for six Streptomyces species: S. griseus strains UI-1158w, NRRL B-599, NRRL 3242, and ATCC 10137, S. punipalus NRRL 3529, and S. lavendulae NRRL B-2036. S. griseus NRRL B-599 yielded complete metabolism of the substrate; therefore, this culture was chosen for preparative-scale fermentation and isolation of the metabolites.

The preparative incubation with 200 mg of N-methylcolchicine amide yielded one major and two minor (phenolic) metabolites. The major metabolite (65%) was unambiguously characterized as colchicine amide (compound V), arising via N-demethylation of the vinylagous amide in the tropoline ring of N-methylcolchicine amide. The NMR spectrum clearly indicated the loss of the N-methyl doublet (at 3.12 ppm in N-methylcolchicine amide) and the appearance of the two amine protons (NH2) at 6.12 ppm (the single amine proton of the substrate, NH-CH3, appears at 4.74 ppm). The mass spectral fragmentation pattern of this metabolite was consistent with the proposed structure. Indeed, NMR and mass spectral data for standard colchicine amide and the metabolite were identical and consistent with the reported spectra (4, 18). In addition, the two samples co-chromatographed on both TLC systems and the high-performance liquid chromatography system described above and gave the same melting point with no depression on admixture.

Characterization of the two phenolic metabolites, VI and VII, was more difficult due to the small amounts of materials available. However, sufficient spectral and chemical evidence was accumulated to suggest that their structures are consistent with 2-O-desmethyl-N-methylcolchicine amide (VI) and 3-O-desmethyl-N-methylcolchicine amide (VII). The phenolic nature of these derivatives was suggested by their partitioning behavior into aqueous base, in contrast to N-methylcolchicine amide and colchicine amide. Mass spectral analysis confirmed that they were monodemethylated derivatives, and the phenolic nature was confirmed by remethylation using diazomethane to yield the starting material, N-methylcolchicine amide. Proton NMR analysis indicated that both the N-methyl group (doublet at ca. 3.12 ppm) and the Cl-methoxy group (at ca. 3.67 ppm, upfield from the other methoxyl signals due to steric interaction with the tropolone ring) were intact in both metabolites. To aid these assignments, an analogy was made with the spectral characteristics of the corresponding phenols of colchicine (compound
II). In the work of Cross et al. (2), as well as that of Schoenharting et al. (15) with 2- and 3-O-desmethylcolchicine, it was observed that replacement of the C3-methoxyl group by a hydroxyl caused the C2-methoxy (originally at 3.93 ppm) to shift to higher field (3.95 to 3.99 ppm). In the case of VII, the observed shift from 3.92 to 4.02 ppm was entirely consistent with this assignment. Furthermore Cross et al. suggested that the replacement of the C2-methoxyl by a hydroxyl shifted the C3-methoxyl to 3.92 ppm, which is precisely where we found the signal in VI. Attempts were also made to further confirm these assignments by evaluating the upfield shift of the C4-aromatic proton upon the addition of base. It was anticipated that the upfield shift caused by the ortho phenolate of VII would be greater than that caused by the meta phenolate of VI (6, 9). Sufficient material for definitive results was only available in the case of VI, but the anticipated shift of 0.31 ppm was observed, consistent with a meta-phenol (6, 9). The chromatographic behavior was also consistent with the corresponding phenols of colchicine. On TLC, higher Rf values were observed for the 2-phenol (VI), probably due to increased steric shielding of the phenol relative to VII, as is observed with the colchicine phenols (7; Davis, unpublished data). High-performance liquid chromatography analysis on the reverse-phase system allowed for elution of the more polar phenol VII (retention time = 8 min) more rapidly than VI (retention time = 10 min), again in analogy with the corresponding colchicine phenols (3).

The N-dealkylation of N-methylcolchicineamide represents the first report of such a metabolic reaction on a tropolone ring system. At the present time, it is unknown whether this reaction occurs in mammals. This biotransformation would be significant in that it may represent detoxification while retaining biological activity as an antineoplastic agent. This suggestion is based on the observation that the product colchicineamide (V) is equipotent to, but one order of magnitude less toxic than, N-methylcolchicineamide (I), as well as colchicine (II) (11).

Heteroatom-alkyl cleavage at the C-10 position of colchicine analogs by using microorganisms has now been described for the N-demethylation of N-methylcolchicineamide to yield colchicineamide, as well as the S-demethylation of thiocolchicine to yield compound IV (17; Roussel-UCLAF, British patent 923,421, Chem. Abstr. 59:13320a, 1963). It has been suggested (8) that the product of colchicine transformation by S. griseus (17; Roussel-UCLAF, British patent 923,421, Chem. Abstr. 59:13320a, 1963) may result from O-demethylation in an analogous fashion, although this question does not appear to have been fully resolved. It is hoped that a more thorough understanding of the metabolism of colchicine derivatives, which may represent bioactivation, bioinactivation, detoxification, or some combination of these (12), will aid in the rational design of new antitumor agents of this structural class.

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