Microbial Transformations and ^{13}C-NMR Analysis of Colchicine

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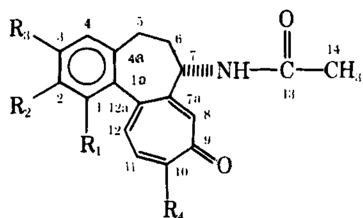
Abstract □ Several microorganisms were screened for their ability to biotransform colchicine, and two were selected for preparative scale fermentations. *Streptomyces spectabilis* and *Streptomyces griseus* both produced O^2-demethylcolchicine and O^3-demethylcolchicine but in different amounts. The ^{13}C-NMR assignments of colchicine, O^{10}-demethylcolchicine, and trimethylcolchicinic acid are reported and were used to help identify the structures of the metabolites.

Keyphrases □ Colchicine—microbial transformations, ^{13}C-NMR spectra, metabolites □ Suppressant (gout)—colchicine, microbial transformations, NMR spectra, metabolites □ *Streptomyces spectabilis*—colchicine metabolism □ *Streptomyces griseus*—colchicine metabolism

Colchicine (I) is one of the oldest drugs used in the treatment of gout. It has no equal for the relief of pain from acute gouty arthritis (1). In spite of the large amount of information available concerning the pharmacology of I, its metabolism and mechanism of action in humans are still unknown.

Several reports (1–3) suggested that a metabolite(s) is (are) the biologically active form of the drug. The only report of a study of human metabolism of I in gout patients suggested that a major portion of I is deacetylated, but whether deacetylcolchicine is a human metabolite has not been established (4). With the modified Undenfriend system, a redox system similar in many respects to liver microsomes, I was converted to O^2-demethylcolchicine (II), O^3-demethylcolchicine (III), O^{10}-demethylcolchicine (colchiceine, IV), and a rearranged tropolone ring derivative given the trivial name colchinal (5). The O^2-, O^3-, and O^{10}-demethylcolchicines also were reported as metabolic products of I using mammalian liver microsomes (6).

While the production and isolation of quantities of drug metabolites from animals or animal tissue homogenates or from conventional synthesis or modification may be extremely difficult, microorganisms possess adaptive enzymes which often can be raised to high levels under suitable culture conditions to produce desired metabolic products. The application of this technique in the biotransformation of numerous classes of compounds has



I: $R_1 = R_2 = R_3 = R_4 = OCH_3$
II: $R_1 = R_3 = R_4 = OCH_3$, $R_2 = OH$
III: $R_1 = R_2 = R_4 = OCH_3$, $R_3 = OH$
IV: $R_1 = R_2 = R_3 = OCH_3$, $R_4 = OH$
VI: $R_2 = R_3 = R_4 = OCH_3$, $R_1 = OH$

been successful, as shown by the appearance of three major "compendia" (7–9). Three reviews (10–12) indicated that microbial systems also can be used to study mammalian metabolism. This paper reports on the ^{13}C-NMR assignments for I and on the isolation and identification, using ^{13}C-NMR spectroscopy, of two microbial metabolites of I.

RESULTS AND DISCUSSION

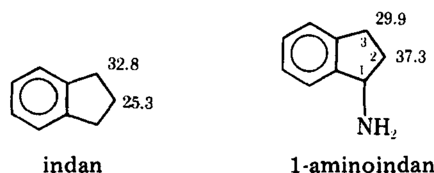
The use of ^{13}C-NMR spectroscopy has become almost routine in structural elucidation studies of natural products as well as of other drugs. ^{13}C-NMR spectroscopy also is particularly well suited for microbial transformation studies since the starting materials are usually available in large quantities, thus allowing detailed studies of ^{13}C-NMR properties. A comparison of the ^{13}C-NMR data of metabolites and starting material will allow ready formulation of the structures of the metabolites.

There are two published ^{13}C-NMR spectra of colchicine (I). The biosynthesis of I was studied using ^{13}C-NMR data, but complete assignments were not made (13). In another report (14), the complete assignments for I were made using model compounds. Close examination of some of these spectral assignments (14) suggested that some assignments may be in error, and this situation prompted a more detailed study of the carbon assignments.

The assignments of C-1a, C-4, C-7, C-8, C-9, C-10, C-11, C-12, C-13, and C-14 (Table I) were made from known chemical shift theory, additivity principles, and model compounds (15–17) as well as from single-frequency off-resonance decoupling (SFORD) experiments and are in agreement with those reported (14). The signals for C-4, C-8, C-11, and C-12 were confirmed by selective single-frequency proton-decoupling experiments[1]. The other carbon assignments listed in Table I for I differ from those reported previously (14), and evidence is presented here to establish their correctness.

There are four methoxyl carbon signals at 61.5, 61.3, 56.6, and 56.3 ppm[2]. When methoxyl groups have di-*ortho* substituents, the chemical shift of these carbon signals appears at a lower field (60–63 ppm) than methoxyl groups that have none or only one *ortho* substituent (55–57 ppm) (19–23). Thus, the methoxyl groups at C-10 and C-3 can be assigned to the signals at 56.3 and/or 56.5 ppm, and those at C-1 and C-2 can be assigned to the signals at 61.3 and/or 61.5 ppm.

The two signals at 30.1 (t) and 36.6 (t) ppm are readily assigned to either C-5 or C-6. The most downfield signal was previously assigned to C-5 (13, 14). The assignments for C-5 and C-6 were made by using indan as a model (14). However, a better model for comparison would be 1-aminoindan whose ^{13}C-NMR data (17) show that C-2 is much farther downfield than the benzylic carbon (C-3), illustrating the deshielding effect of an amino group at C-2 (β effect) and shielding at C-3 (γ effect). A similar relationship as exists in 1-aminoindan exists in I, and thus the amide would be expected to deshield C-6 relative to C-5. Thus, the as-



[1] The protons H-4, H-8, H-11, and H-12 were assigned in the ^{1}H-NMR spectrum of I (18).

[2] The authors of Ref. 14 reported only one signal at 61.3 and three signals at 56.1, 56.4, and 56.4. The authors of Ref. 13 reported two at 61.2 and 61.0 and two at 55.8 and 56.1, which are in close agreement with the signals listed in Table I.

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Table I—^{13}C-NMR Data for I–V [a]

Assignment [b]	I	II	III	IV [c]	V [d]	V·HCl [e]
C-1	153.8 s^{1}	147.8 s^{1}	150.3 s^{1}	153.9 s^{1}	153.7 s^{1}	155.2 s^{1}
C-2	142.2 s	138.1 s	139.3 s	141.8 s	141.6 s	141.6 s^{2}
C-3	151.4 s^{1}	145.0 s^{1}	149.9 s^{1}	151.1 s^{1}	153.3 s^{1}	150.7 s^{1}
C-4	107.9 d	107.0 d	110.3 d	107.7 d	107.3 d	108.2 d
C-5	30.1 t	29.8 t	29.7 t	29.9 t	30.6 t	29.6 t
C-6	36.6 t	36.9 t	36.6 t	37.6 t	41.8 t	37.0 t
C-7	52.8 d	52.8 d	52.8 d	52.9 d	53.7 d	54.7 d
C-8	130.7 d	130.7 d	130.7 d	119.5 d	118.6 d	124.8 d^{3}
C-9	179.6 s	179.7 s	179.7 s	170.1 s^{2}	172.2 s^{2}	168.9 s^{4}
C-10	164.3 s	164.3 s	164.2 s	170.2 s^{2}	168.7 s^{2}	168.3 s^{4}
C-11	113.1 d	112.8 d	112.9 d	122.5 d	123.8 d	126.7 d^{3}
C-12	134.5 d	135.1 d	135.3 d	141.6 d	141.6 d	144.7 d^{3}
C-13	170.0 s	170.0 s	170.2 s	170.5 s^{2}	—	—
C-14	22.7 q	22.9 q	22.8 q	22.8 q	—	—
C-1a	126.0 s	125.1 s	125.1 s	126.1 s	126.0 s	124.0 s
C-4a	134.4 s	129.9 s	134.2 s	134.6 s	135.7 s^{3}	134.3 s
C-7a	152.6 s	152.2 s	152.5 s	151.7 s	150.6 s^{1}	148.7 s^{1}
C-12a	137.2 s	136.9 s	137.1 s	136.5 s	135.6 s^{3}	141.5 s^{2}
C-1OCH$_3$	61.3 q^{2}	61.3 q	61.3 q^{2}	61.3 q^{3}	61.1 q^{4}	61.7 q^{5}
C-2OCH$_3$	61.5 q^{2}	—	61.5 q^{2}	61.5 q^{3}	60.9 q^{4}	61.2 q^{5}
C-3OCH$_3$	56.3 q^{3}	56.3 q^{2}	—	56.3 q	56.2 q	56.3 q
C-10OCH$_3$	56.5 q^{3}	56.5 q^{2}	56.4 q	—	—	—

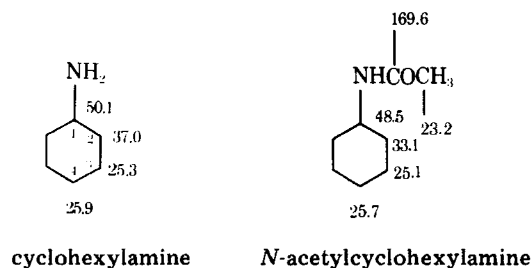
[a] Run in deuterochloroform. [b] Assignments are based on predicted chemical shifts, comparisons with literature data, single-frequency off-resonance decoupling, selective proton-decoupling, and proton-coupled data. Assignments bearing the same numerical superscript in any one spectrum may be reversed. [c] Selective single-frequency proton-decoupling was done to confirm C-8, C-11, and C-12. However, since the H-11 and H-12 proton assignments are not unambiguously known (Ref. 18, p. 421), the C-11 and C-12 signals could be reversed, but the ones listed are preferred since it is unlikely that C-11 would change 28.5 ppm downfield (I → IV) and C-12 would change 12.9 ppm upfield (I → IV), as would be the case if the signals were reversed. The assignments of the 151.7-ppm signal to C-7a and the 141.8-ppm signal to C-2 followed from selective single-frequency proton-decoupling experiments at low decoupling power (irradiation at δ_H3.9). The proton-coupled spectrum was also recorded, and the multiplicities of the signals for C-4a and C-12a were the same as in I. [d] The assignments listed for V are based on analogy to IV and off-resonance experiments only. [e] A few drops of methanol-d_4 were added to increase solubility. Significant shifts have occurred between the amine (V) and its hydrochloride, making assignments somewhat ambiguous without selective single-frequency proton-decoupling and proton-coupled data, which were not obtained.

signments of the signal at 36.6 ppm to C-6 and at 30.1 ppm to C-5 in I would seem more appropriate.

The correctness of these assignments was established by several additional observations. To evaluate the effect of an amine *versus* an amide, the ^{13}C-NMR spectrum of trimethylcolchicinic acid (V) was obtained. It showed the triplet signals (off resonance) at 30.6 (C-5) and 41.8 (C-6) ppm. This downfield shift of about 5 ppm for C-6 is comparable to that observed in cyclohexylamine[3] *versus* *N*-acetylcyclohexylamine. It also has been reported that the C-2 carbon in cyclohexylamine shifts to a higher field (7.1 ppm) in the hydrochloride salt while the C-3 carbon shifts upfield by only 1.5 ppm[4]. Similarly, the hydrochloride salt of trimethylcolchicinic acid (V·HCl) was prepared and showed upfield shifts of 1.0 ppm for C-5 (29.6 ppm) and 4.8 ppm for C-6 (37.0 ppm).

The remaining unassigned signals in I (C-1, C-2, C-3, C-4a, C-7a, and C-12a) were assigned by interpretation of the proton-coupled ^{13}C-NMR spectral data and selective single-frequency proton-decoupling experiments. The use of long-range ^{13}C–H coupling is becoming increasingly important in making unambiguous carbon assignments, particularly for quaternary carbons in aromatic compounds; a number of empirical observations have become evident. Three-bond coupling constants ($^3J_{C\text{-}H}$) are always larger (4–10 Hz) than two- or four-bond coupling constants (usually <4 Hz) (24–26).

The proton-coupled spectrum of I showed the signal at 137.2 ppm as a broad triplet ($^3J_{C\text{-}H}$ = 8 Hz) and was assigned to C-12a since there are apparently two equal three-bond couplings (H-8 and H-11). The signal at 142.2 ppm was complex and was assigned to C-2 on the basis that C-2 would be expected to be either a pentet (equal coupling constants to H-4 and OCH$_3$) or an overlapping doublet of quartets (unequal coupling



cyclohexylamine　　*N*-acetylcyclohexylamine

[3] The assignments for cyclohexylamine were reported on p. 153 of Ref. 16. The chemical shift values reported (on the structure) are those obtained on the instrument used in the present study.
[4] See p. 153 of Ref. 16.

constants to H-4 and OCH$_3$). The 134.4-ppm signal was a broad singlet and was assigned to C-4a.

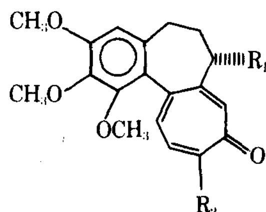
The correctness of these assignments, as well as the assignments for C-1, C-3, and C-7a, was confirmed by carrying out a selective single-frequency proton-decoupling experiment[5]. Irradiation at δ_H3.9 (OCH$_3$) showed the 142.2-ppm signal (C-2) as a doublet (three-bond coupling to H-4) and the 153.8- and 151.4-ppm signals (C-1 and/or C-3) as sharp singlets. The signal at 152.6 ppm appeared as a doublet and was assigned to C-7a[6] (three-bond coupling to H-12). The other signals (134.5 and 137.2) were unchanged. Irradiation at δ_H7.6 (C=CH) showed the signal at 137.2 ppm as a singlet, clearly identifying it as C-12a; the signals at 142.2 (C-2) and 134.4 (C-4a) were essentially the same as in the proton-coupled spectrum.

Finally, irradiation at δ_H2.5 (—*CH*$_2$—*CH*$_2$—) showed a sharp singlet for the 134.4-ppm signal while the 142.2 and 137.2 signals remained the same as in the proton-coupled spectrum. This sharpening results from eliminating the two-bond coupling of C-4a to the protons at C-5. Also, in this last experiment, the C-1a (126.0) signal appeared as a double doublet[7] (unequal three-bond coupling to H-4 and H-12). These experiments provided evidence that the assignments listed in Table I for C-1, C-2, C-3, C-4a, C-7a, and C-12a are correct. Thus, the complete assignments of the carbon signals in the ^{13}C-NMR spectrum of I have been made.

After preliminary screening of I for biotransformation products using several microorganisms in different media (Table II), only *Streptomyces spectabilis* (ATCC 27465) and *Streptomyces griseus* (ATCC 13968) (both grown in medium *θ*) were capable of metabolizing I as indicated by TLC. The other microorganisms did not show any ability to metabolize I. Incubation of I with stirred cultures of *S. spectabilis* showed that two more polar metabolites were being produced. These metabolites were very difficult to separate. The separation was accomplished by preparative TLC using 4% ethanol in chloroform on alumina impregnated with sodium hydroxide. With this procedure, both metabolites were obtained in pure form.

The ^{13}C-NMR spectra of the two metabolites (A and B) showed only three methoxyl carbon signals, immediately suggesting mono-*O*-de-

[5] This experiment was conducted at low decoupling power such that in addition to one-bond coupling, long-range ^{13}C-H is also evident, although the splittings are no longer the true coupling constants (27).
[6] C-7a would be expected to be the most deshielded nonoxygenated carbon signal in colchicine since it is the *β*-carbon of an *α*,*β*-unsaturated carbonyl system and also experiences the *β* effect of the amide group. The 152.6-ppm signal was masked in the proton-coupled spectrum by the 153.8- and 151.4-ppm signals.
[7] The 126.0-ppm signal was obscured in the proton-coupled spectra (C-8 doublet) and appeared as a broad unresolved signal upon irradiation at δ_H3.9 and δ_H7.6.



V: $R_1 = NH_2$, $R_2 = OH$
VII: $R_1 = NH_2$, $R_2 = OCH_3$
VIII: $R_1 = {=}O$, $R_2 = OCH_3$

methylated derivatives of I. O^{10}-Demethylcolchicine (colchiceine, IV), readily prepared by hydrolysis (0.1 *N* HCl) of I (28, 29), was not one of the metabolites (TLC), which left three possibilities: O^3-demethylcolchicine (III), O^2-demethylcolchicine (II), and O^1-demethylcolchicine[8]. The ^{13}C-NMR data for Metabolite A showed one methoxyl signal at 56.4 ppm and two at 61.5 and 61.3 ppm. Since the methoxyl carbon signals resonating near 56 ppm in I have been assigned to C-3 and/or C-10 and since Metabolite A does not correspond to O^{10}-demethylcolchicine (IV), it must be III. The other signals in the ^{13}C-NMR spectrum of Metabolite A are also in agreement (Table I). The ^{1}H-NMR spectrum of Metabolite A also was in agreement with that reported for III (5), and a direct comparison with an authentic sample confirmed the identity of Metabolite A as III.

The ^{13}C-NMR data for Metabolite B showed two methoxyl signals at 56.5 and 56.3 ppm (OCH_3 at C-3 and/or C-10) and one at 61.3 ppm, indicating that Metabolite B is either VI or II. The ^{1}H-NMR spectrum of Metabolite B showed a methoxyl group at δ_H3.64. Since a methoxyl group appearing near δ 3.6 in I derivatives has been assigned to C-1 (18), this would eliminate VI. The ^{1}H-NMR data were in agreement with those reported for II (5), and a direct comparison with an authentic sample confirmed the identity of Metabolite B as II.

The soil bacterium, *Arthobacter colchovorum*, metabolizes I to 7-deacetylcolchicine (VII) and 7-deacetamino-7-oxocolchicine (VIII) (30). One other report of the microbial metabolism of I using *S. griseus* has appeared, but the metabolite's structure was established only as a monodemethylated colchicine derivative (31, 32). However, it has been reported that this metabolite is VI (30). On the other hand, another report (9) suggested that this metabolite is probably IV.

When I was added to shaken cultures of *S. griseus* (ATCC 13968) using the fermentation conditions reported previously (32), two metabolites were observed. Subsequent preparative scale fermentations resulted in the isolation of mostly II and a small amount of III. The overall yields using *S. griseus* were lower than with *S. spectabilis*. Furthermore, II was the major product with *S. griseus* whereas III was the major product with *S. spectabilis*.

EXPERIMENTAL[9]

Fermentation Procedures—Stock cultures of microorganisms were maintained on mycophil (BBL) agar (fungi) or Eugonagar (bacteria). Preliminary biotransformation studies were carried out on gyrotory shakers operating at 200 rpm (room temperature) in erlenmeyer flasks holding one-fifth their volume of medium. A two-stage fermentation procedure was utilized in which a sterile liquid culture medium was inoculated with the surface growth from agar slants. These stage I cultures were incubated for 24 hr (200 rpm, room temperature). Two milliliters of the stage I culture broth was used as inoculum for stage II cultures. The substrate was added to 24-hr stage II cultures as a 10% ethanolic solution at a concentration of 0.1 mg/ml of stage II medium.

Fermentation Medium α consisted of: dextrose, 20 g; yeast extract, 5 g; peptone, 5 g; NaCl, 5 g; K_2HPO_4, 5 g; and H_2O, 1000 ml.

Fermentation Medium β consisted of: NH_4NO_3, 4 g; KH_2PO_4, 1.5 g; Na_2HPO_4, 1.5 g; $MgSO_4$, 0.2 g; $FeSO_4{\cdot}7H_2O$, 0.05 g; $MnSO_4{\cdot}H_2O$, 0.002 g; $CaCl_2{\cdot}H_2O$, 0.01 g; yeast extract, 0.5 g; and H_2O, 1000 ml.

Fermentation Medium γ consisted of: KH_2PO_4, 0.7 g; K_2HPO_4, 0.7 g; $MgSO_4$, 0.7 g; NH_4NO_3, 1.0 g; NaCl, 0.005 g; $FeSO_4{\cdot}7H_2O$, 0.002 g; $ZnSO_4$, 0.002 g; $MnSO_4{\cdot}H_2O$, 0.001 g; glucose, 2 g; and H_2O, 1000 ml.

Table II—Microorganisms and Media Used for Screening of Colchicine

Organism	Medium			
Alternaria solani (11078)	α	δ	ϵ	θ
Arthrobacter simplex (6946)	β	δ		θ
Arthrobacter sp. (19140)	β	δ		θ
Arthrobacter sp. (21237)	β	δ		θ
Aspergillus flavipes (1030)	α	δ	ϵ	θ
Aspergillus flavipes (11013)	α	δ	ϵ	θ
Aspergillus flavipes (16795)	α	δ	ϵ	θ
Aspergillus flavus (9170)	α	δ	ϵ	θ
Aspergillus flavus (24741)	α	δ	ϵ	θ
Aspergillus niger (10548)	α	δ	ϵ	θ
Aspergillus niger (11394)	α	δ	ϵ	θ
Aspergillus niger (16888)		δ	ϵ	
Aspergillus ochraceus (18500)	α	δ	ϵ	θ
Aspergillus ochraceus (22947)	α	δ	ϵ	θ
Aspergillus parasiticus (15517)	α	δ	ϵ	θ
Beauvaria bussiana (13144)	α	δ	ϵ	θ
Botrytis allii (9435)	α	δ	ϵ	θ
Calonectria decora (14767)	α	δ	ϵ	θ
Chaetomium cochloides (10195)	α	δ	ϵ	θ
Cladosporium resinae (22712)	α	δ	ϵ	θ
Cladosporium resinae f. *avelleneum* (22711)	α	δ	ϵ	θ
Cunninghamella blakesleeana (8688a)	α	δ	ϵ	θ
Cunninghamella echinulata (NRRL 3655)	α	δ	ϵ	θ
Cunninghamella echinulata (9244)	α	δ	ϵ	θ
Cunninghamella echinulata (11585a)	α	δ	ϵ	θ
Cunninghamella echinulata (11585b)	α	δ	ϵ	θ
Cunninghamella elegans (9245)	α	δ	ϵ	θ
Curvularia lunata (13633)	α	δ	ϵ	θ
Curvularia lunata (12017)	α	δ	ϵ	θ
Cylindrocarpon radicicola (11011)	α	δ		θ
Fomes pinicola (15341)	α	δ	ϵ	θ
Fusarium javanicum (12575)	α	δ	ϵ	θ
Fusarium oxysporum (7601)	α	δ	ϵ	θ
Fusarium oxysporum (26297)	α	δ	ϵ	θ
Fusarium oxysporum f. sp. *cepue* (11711)	α	δ	ϵ	θ
Fusarium oxysporum f. sp. *gladioli* (11137)	α	δ	ϵ	θ
Fusarium oxysporum f. sp. *tulipae* (15652)	α	δ	ϵ	θ
Fusarium solani var. *coeruleum* (24389)	α	δ	ϵ	θ
Mucor griseo-cyanus (1207)	α	δ	ϵ	θ
Nocardia corallinia (19070)				θ
Nocardia corallinia (19071)				θ
Nocardia corallinia (19148)				θ
Nocardia minima (19050)				θ
Penicillium adametzi (10407)	α	δ		θ
Penicillium chrysogenum (9480)	α	δ		θ
Penicillium chrysogenum (10002)	α	δ		θ
Penicillium frequentans (10444)	α	δ		θ
Penicillium oxalicum (24784)	α	δ		θ
Penicillium spinulosum (16348)	α	δ		θ
Rhizopus arrhizus (11145)	α	δ		θ
Rhizopus stolonifer (6227b)	α	δ		θ
Rhizopus stolonifer (15441)	α	δ		θ
Septomyxa affinis (6737)	α	δ		θ
Streptomyces griseus (10137)	α	δ		θ
Streptomyces griseus (13968)	α	δ	β	θ
Streptomyces griseus (23337)		δ	β	θ
Streptomyces purpurascens (21326)		δ	β	θ
Streptomyces roseochromogenus (13400)		δ	β	θ
Streptomyces sp. (15077)		δ	β	θ
Streptomyces spectabilis (27465)		δ	β	θ
Syncephalastrum racemosum (18192)	α	δ		θ
Whetzelinia sclerotiorum (18015)	α	δ		θ
Whetzelinia sclerotiorum (24156)	α	δ		θ

[8] The ^{13}C-NMR data for IV are also recorded in Table I and show marked differences to I–III, particularly for those signals located in the tropolone ring (C-8, C-9, C-10, C-11, and C-12).

[9] Melting points were determined on a Thomas-Hoover apparatus and are uncorrected. IR spectra were run in potassium bromide using a Beckman IR-33 spectrophotometer. ^{1}H-NMR and ^{13}C-NMR spectra were recorded in deuterochloroform on a Jeol-FX60 Fourier-transform NMR spectrometer with tetramethylsilane as the internal standard. *N*-Acetylcyclohexylamine was prepared by acetylation of cyclohexylamine. Colchicine USP was purchased from Inverni & Della Beffa, Milan, Italy. Trimethylcolchicinic acid (V) was obtained commercially (K & K Laboratories-ICN) or was prepared by hydrolysis of I according to the published procedure (28), mp 156–158° [lit. (28, 29) mp 160–162°]. The hydrochloride salt was prepared, mp 202–204° [lit. (29) mp 191°]. O^{10}-Demethylcolchicine (IV) (colchiceine) was prepared by hydrolysis (0.1 *N* HCl) according to published procedures (29, 30), mp 175–177° [lit. (29) mp 178–179°].
^{13}C-NMR (15.03 MHz) data were recorded using a 4000-Hz spectral width, a 45° pulse, 8K data points, and a repetition rate of 10 sec. All proton resonances were decoupled by a broad band (1 kHz) irradiation for proton noise-decoupled spectra. The single-frequency off-resonance experiments were conducted by centering the decoupling frequency 1100 Hz downfield from tetramethylsilane. The selective single-frequency proton-decoupled experiments were carried out by centering the decoupling frequency on the appropriate proton resonance and recording the spectra at several power settings. The proton-coupled spectra were recorded using the gated decoupling mode (decoupler off during data acquisition) and 16K data points.

Fermentation Medium δ consisted of: dextrose, 20 g; yeast extract, 5 g; soytone, 5 g; NaCl, 5 g; K_2HPO_4, 5 g; and H_2O, 1000 ml.

Fermentation Medium ε consisted of: dextrose, 30 g; KH_2PO_4, 1.0 g; K_2HPO_4, 2.0 g; soytone, 10.0 g; $MgSO_4$, 0.5 g; $NaNO_3$, 2.0 g; $FeSO_4$, 0.02 g; KCl, 0.5 g; and H_2O, 1000 ml.

Fermentation Medium θ consisted of: dextrose, 10 g; cornsteep liquor, 10 g; soya flour, 10 g; dry malt extract, 5 g; $CaCO_3$, 1 g; NaCl, 5 g; and H_2O, 1000 ml.

Fermentation Sampling—The fermentations were sampled by withdrawing 5 ml of culture broth, adjusting to pH 7, and extracting with 3 × 5 ml of chloroform. The chloroform layers were evaporated to dryness, and the residues obtained were redissolved in chloroform and spotted on precoated silica gel G TLC plates. The plates were developed in 8% methanol in benzene–ethyl acetate–diethylamine (5:4:1) and were visualized with UV light and by spraying with 10% HCl, heating for 1–2 min, and spraying with 5% $FeCl_3$ in 0.5 *N* HCl.

Preparative Scale Biotransformation of Colchicine (I) Using *S. spectabilis*—*S. spectabilis* was grown in 5 liters of Medium θ contained in a 7.5-liter fermentor jar[10]. After a 24-hr incubation, I (500 mg/5 ml of absolute ethanol) was added to the culture; the incubation (200-rpm stir rate, 2-liters/min air flow rate, room temperature) was continued for 14 days. The culture was filtered through a buchner funnel, and the aqueous culture broth was adjusted to pH >12 with potassium hydroxide and extracted with 12 × 500 ml of chloroform. The combined chloroform layers were dried over sodium sulfate and evaporated *in vacuo* (40°) to leave 632 mg of a yellow residue, shown by TLC [8% methanol in benzene–ethyl acetate–diethylamine (5:4:1), silica gel G] to consist mostly of I.

The aqueous basic layer was adjusted to pH 4–5 with acetic acid and extracted with 12 × 500 ml of chloroform. The combined chloroform layers were dried over sodium sulfate and evaporated *in vacuo* (40°) to leave 104 mg of a yellow-brown oily residue. This residue was applied to two precoated alumina plates (1 mm), which had been sprayed with 5% aqueous NaOH and dried for 10 min at 100°. Multiple development of the plate in 4% ethanol–chloroform (six times) separated the metabolites into two distinct bands (R_f 0.65 and 0.58).

The lower R_f band was located under UV light, scraped, and eluted with 20% methanol–chloroform to yield 39 mg of Metabolite A after solvent removal. This metabolite could not be induced to crystallize. The material (amorphous powder) was pure by TLC; IR: ν_{max} ($CHCl_3$) 3260 (broad), 3525, 3445, 2950, 1683, 1620, 1594, and 1555 cm^{-1}; ^1H-NMR ($CDCl_3$): δ 1.98 (3H, s, $NHCOCH_3$), 2.4 (4H, m, H-5,6), 3.64 (3H, s, C-1 OCH_3), 4.00 (6H, s, C-2, 10 OCH_3), 4.58 (1H, m, H-7), 6.58 (1H, s, H-4), 6.87 (1H, d, J = 11 Hz, H-11), 7.36 (1H, d, J = 11 Hz, H-12), 7.62 (1H, s, H-8), and 8.05 (1H, m, $NHCOCH_3$) ppm. A direct comparison of Metabolite A with an authentic sample of O^3-demethylcolchicine (III) showed that they had the same behavior on TLC (co-TLC), superimposable IR spectra, and the same ^1H-NMR spectra. The ^{13}C-NMR data for Metabolite A (III) are listed in Table I.

Extraction of the higher R_f band (0.65) in the same manner gave 20 mg of Metabolite B. Crystallization from chloroform–hexane afforded 4 mg of B as yellow needles, mp 184–186°; IR: ν_{max} (KBr) 3290 (broad), 2950, 1664, 1618, 1590, and 1550 cm^{-1}; ^1H-NMR ($CDCl_3$): δ 1.97 (3H, s, $NHCOCH_3$), 2.4 (4H, m, H-5,6), 3.64 (3H, s, C-1 OCH_3), 3.93 (3H, s, OCH_3), 4.02 (3H, s, OCH_3), 4.16 (1H, m, H-7), 6.53 (1H, s, H-4), 6.85 (1H, d, J = 11 Hz, H-11), 7.37 (1H, d, J = 11 Hz, H-12), 7.59 (1H, s, H-8), and 7.82 (1H, m, $NHCOCH_3$) ppm. A direct comparison of Metabolite B with an authentic sample of O^2-demethylcolchicine (II) showed no mixed melting-point depression, the same behavior on TLC (co-TLC), superimposable IR spectra (KBr), and the same ^1H-NMR spectra. The ^{13}C-NMR data for Metabolite B (II) are listed in Table I.

Preparative Scale Biotransformation of I Using *S. griseus*—*S. griseus* (ATCC 13968) was grown in 5.0 liter of Medium θ contained in 50 500-ml erlenmeyer flasks. After a 24-hr incubation, 500 mg of I was evenly distributed among the cultures; incubation was continued for 12 days (300 rpm, 26°). The aqueous culture broth was adjusted to pH >12 with 1.0 *M* NaOH and then extracted with chloroform. The chloroform extract was dried over sodium sulfate and then evaporated to leave a brown oily residue (750 mg), which was shown by TLC to consist of starting material but not of metabolites.

The aqueous basic layer was adjusted to pH 5 with acetic acid and extracted with chloroform. The chloroform extract was dried over sodium sulfate and evaporated to give a yellow oily residue (85 mg), which was applied to an alumina plate (1.0 mm) impregnated with 5% NaOH and dried at 100° for 10 min. The plate was developed (four times) in 4% ethanol–chloroform, and the higher R_f band was located under UV light, scraped, and eluted with acetone to give a yellow crystalline residue (38 mg). Crystallization from chloroform–hexane afforded 26 mg of II, mp 185–187°, identical in all respects with authentic II [melting point, mixed melting point, TLC, co-TLC, ^1H-NMR, and superimposable IR spectra (KBr)].

Extraction of the lower R_f band in the same manner yielded 4 mg of III, which had the same mobility on TLC (co-TLC) as authentic III. The ^1H-NMR data clearly revealed two methoxyl signals at δ 4.00 and one at δ 3.64 and were essentially identical to those of authentic III.

[10] New Brunswick model FS307.

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Urinary Excretion of Chlorpheniramine and Pseudoephedrine in Humans

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Abstract □ A specific high-pressure liquid chromatographic method for the determination of chlorpheniramine and pseudoephedrine in urine was developed and applied in a urinary excretion study of normal healthy subjects who received a sustained-release dosage form containing 8 mg of chlorpheniramine maleate and 120 mg of pseudoephedrine hydrochloride. Five subjects received one dose on Day 1, followed by multiple dosing every 12 hr for 7 days without ammonium chloride administration. Four subjects received one dose of the sustained-release dosage form together with ammonium chloride. Urine samples were collected during the 1st day and at steady state. The method is specific and simultaneously determines chlorpheniramine, two metabolites (mono- and didesmethylchlorpheniramine), pseudoephedrine, and norpseudoephedrine. The assay recovery was >97% (0.06–3 μg/ml) for chlorpheniramine maleate and >98% (1.5–75 μg/ml) for pseudoephedrine hydrochloride. Excretion of chlorpheniramine and its two metabolites in urine was enhanced after ammonium chloride administration. At steady state, a change in urine pH from 5.69 to 6.46 resulted in more than a 25% decrease in chlorpheniramine and monodesmethylchlorpheniramine excretion. In spite of expected changes in its biological half-life, the overall amount of unchanged pseudoephedrine excreted in urine was not affected by urine pH, presumably because it is primarily excreted in urine as intact drug.

Keyphrases □ Chlorpheniramine—urinary excretion, humans, high-pressure liquid chromatographic analysis □ Chlorpheniramine metabolites—urinary excretion, humans, high-pressure liquid chromatographic analysis □ Pseudoephedrine—urinary excretion, humans, high-pressure liquid chromatographic analysis □ Nasal decongestants—chlorpheniramine and pseudoephedrine, urinary excretion, humans □ High-pressure liquid chromatography—analysis, chlorpheniramine and pseudoephedrine in human urine

Chlorpheniramine and pseudoephedrine, two weak basic drugs, are often used together for treatment of nasal congestion. The extent and rate of urinary excretion of the unchanged drugs are pH and urine flow dependent (1–3). Chlorpheniramine is extensively metabolized *via* *N*-dealkylation and excreted as both mono- and didesmethyl compounds (1, 4–6), and it is expected that the excretion of these metabolites in urine is also urine pH dependent. Pseudoephedrine is primarily excreted unchanged in urine (7).

A GLC method was reported for the determination of chlorpheniramine in urine (2). Similarly, methods utilizing GLC (8, 9) or a radiolabeled technique (3) were reported for the determination of pseudoephedrine in urine. The purposes of this investigation were: (*a*) to develop a simple, specific, high-pressure liquid chromatographic (HPLC) method for the simultaneous determination of chlorpheniramine, its demethylated metabolites, and pseudoephedrine and (*b*) to apply this method in studying the urinary excretion of these drugs and their metabolites in humans.

EXPERIMENTAL

Protocol—Nine healthy, nonobese, male subjects participated. They did not receive any drugs, including enzyme-inducing agents and monoamine oxidase inhibitors, 1 month before and during the study. Caffeine-containing and alcoholic beverages also were withheld during the entire study.

Five of the subjects received one sustained-release capsule[1] containing 8 mg of chlorpheniramine maleate and 120 mg of pseudoephedrine hydrochloride on Day 1, after an overnight fast, followed by an additional 4-hr fasting period. Thereafter, the subjects received one capsule every 12 hr for 6 days (Days 2–7) and then one capsule on Day 8. Cumulative urine samples were collected during the 0–24-hr period and at the steady state during the 156–168- and 168–180-hr periods.

The other four subjects received one single capsule with and without ammonium chloride treatment according to a double crossover design. A washout period of 1 week was allowed between treatments. The capsule was always taken after an overnight fast and followed by 4 hr of fasting. One day before and throughout the study period (~68 hr), two enteric coated ammonium chloride tablets[2] (500 mg each) were administered every 2 hr. The first dose taken before retiring and that given on arising always consisted of four tablets (2 g). When a subject's urinary pH was >5.3, one additional ammonium chloride tablet was administered after the urinary pH measurement. If the urinary pH was <4.9, the next scheduled dose was omitted. An attempt was made to control the urine flow by frequent oral administration of liquids throughout the study periods. Urine samples were collected from −1 to 0 hr, *i.e.*, just before the drug was administered, and at frequent intervals up to 48 hr.

During the entire investigation, standard meals were given to all subjects. Urine pH was determined at room temperature, and the volume of each urine sample was measured in a graduated cylinder. An aliquot of 30 ml of the thoroughly mixed sample was withdrawn and stored at −15° until assayed.

Assay—Chlorpheniramine and pseudoephedrine concentrations were determined according to the following methods.

Method I—Two-milliliter samples of urine or standard solution containing 0.06–3 μg of chlorpheniramine maleate[3]/ml and 1.5–75 μg of pseudoephedrine hydrochloride[4]/ml were taken for assay. To the samples were added 0.5 ml of 5% KOH solution and 5 ml of an extraction solvent consisting of hexane–methylene chloride (60:40). The samples were shaken for 30 min and centrifuged. Four milliliters of the solvent was pipetted into a vial containing 0.5 ml of chlorpromazine hydrochloride[5], 1 μg/ml in methanol. The mixture was evaporated under nitrogen. The residue was dissolved in 50 μl of methanol from which 20 μl was removed for injection.

Method II—Two-milliliter samples of urine or standard solution

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