

Synthesis of (3'R,5'S)-*trans*-3'-Hydroxycotinine, a Major Metabolite of Nicotine. Metabolic Formation of 3'-Hydroxycotinine in Humans Is Highly Stereoselective

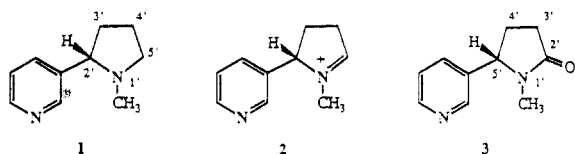
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A method for the synthesis of (3'R,5'S)-*trans*-3'-hydroxycotinine, a major metabolite of nicotine in humans, is described. The method involves deprotonation of (S)-cotinine with lithium diisopropylamide (LDA) followed by oxidation with the transition metal peroxide oxodiperoxymolybdenum(pyridine)(hexamethylphosphoric triamide) (MoOPH) to give an 80:20 mixture of *trans*-/*cis*-3'-hydroxycotinine. The pure (>98%) *trans* isomer is obtained by conversion to the solid hexanoate ester, recrystallization, and cleavage of the ester by heating with *n*-butylamine. GC-MS analysis of urine extracts from several smokers indicated that in humans metabolic 3'-hydroxycotinine is 95-98% *trans*.

The major route of metabolism of the tobacco alkaloid (S)-nicotine (1) in humans and in most mammalian species is a cytochrome P-450 catalyzed oxidation at the 5'-carbon atom. This produces nicotine $\Delta^{1(5)}$ -iminium ion 2,¹ which



is converted to the γ -lactam derivative cotinine (3) in a reaction catalyzed by a cytoplasmic aldehyde oxidase.² Metabolic conversion of nicotine to cotinine in humans has been shown to occur without significant racemization.³ We have estimated that about 70% of the nicotine absorbed by smokers is metabolized via the cotinine pathway.⁴ Further metabolism of cotinine is extensive, with only about 10% appearing in the urine unchanged.⁴⁻⁶ Consequently, cotinine metabolites must account for a substantial proportion of the nicotine absorbed by smokers.

A number of cotinine metabolites have been characterized, but very little data on their quantitative importance in humans are available.^{4,7} McKennis and co-workers reported in the 1960s that a "hydroxycotinine" was a human metabolite of cotinine⁸ and present in the urine of smokers.³ The metabolite isolated from urine was found to be optically active, $[\alpha]_{29}^{20} = +42.5^\circ$, and by conversion to (S)-cotinine it was shown that the hydroxylation had occurred with retention of configuration at the 5'-carbon atom. A metabolite of virtually identical melting point and optical rotation was obtained from a dog following (S)-cotinine administration.⁸ Synthesis of the metabolite suggested that the structure was 3'-hydroxycotinine, but since one of the synthetic steps allowed the possibility of a rearrangement, it was not possible to establish rigorously the position of the hydroxy group.³ Subsequently, Dagne and Castagnoli isolated a hydroxylated metabolite of cotinine from a rhesus monkey following intravenous infusion of cotinine.⁹ This metabolite, which had properties consistent with those of the hydroxycotinine isolated by McKennis et al., was shown to be *trans*-3'-hydroxycotinine by comparison with an authentic sample obtained by total synthesis. Although the material synthesized by Dagne and Castagnoli was racemic, the fact that McKennis et al. had shown that the human metabolite had the *S* configuration at the 5'-carbon makes it reasonable to assume that the configuration of the human metabolite is *trans*-3'R,5'S.

However, in both of these studies the hydroxycotinine isolated from urine was subjected to a number of purification steps, including recrystallization, allowing the possible loss of the *cis* isomer if some were present.

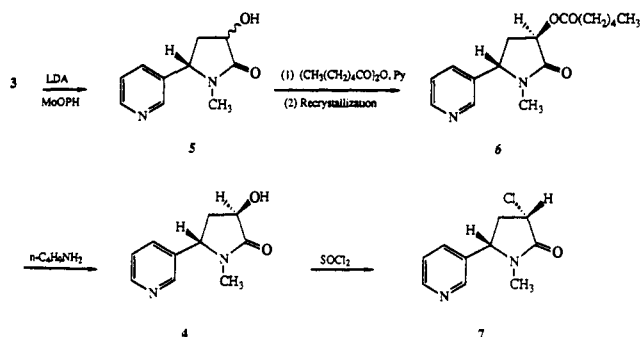
Recently, we and others have found that concentrations of *trans*-3'-hydroxycotinine in smokers' urine exceed cotinine concentrations by 2-3-fold.^{4,10} Since about 10% of the nicotine absorbed by smokers can be accounted for as cotinine excreted in the urine,⁴ 3'-hydroxycotinine is a major nicotine metabolite. In order to carry out pharmacokinetic studies, we required sufficient quantities of the metabolite with the natural stereochemical configuration for administration to human subjects. In this paper we report a synthesis of *trans*-(3'R,5'S)-3'-hydroxycotinine (4) and present chromatographic evidence that the formation of this metabolite in humans is highly stereoselective.

Chemistry

The published methods for the synthesis of 3'-hydroxycotinine are multistep procedures^{3,9} from which the 3'R,5'S stereoisomer can be obtained only following a resolution step. Consequently, we felt that it would be desirable to develop a synthesis starting from readily available (S)-cotinine in order to provide sufficient quantities of the natural stereoisomer for pharmacologic and metabolic studies. A further advantage for using cotinine as the starting material is applicability to the synthesis of isotopically labeled metabolite, since methods for the synthesis of various isotopically labeled analogues of (S)-cotinine are available.¹¹⁻¹⁴

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Scheme I. Synthesis of (3'R,5'S)-trans-3'-Hydroxycotinine and Its Conversion to (3'S,5'S)-cis-3'-Chlorocotinine


Conversion of carbonyl compounds to α -hydroxy derivatives has been accomplished by various methods, including oxidation of enolate anions with molecular oxygen¹⁵ or with peroxides.^{16,17} We found that deprotonation of cotinine with lithium diisopropylamide (LDA) followed by reaction with molecular oxygen gave 3'-hydroxycotinine in modest yield. By capillary GC analysis (described below), it was shown that the product consisted of a 60:40 mixture of trans/cis isomers. This result was encouraging in that it demonstrated the feasibility of direct oxidation of cotinine and that the desired trans isomer predominated. It was reasoned that use of an oxidizing agent with greater steric bulk than oxygen might provide an even greater proportion of the trans isomer, since such a reagent would be expected to attack the enolate anion largely from the side opposite the pyridine ring. Vedejs and co-workers¹⁶ have demonstrated the utility of a transition-metal peroxide, oxodiperoxymolybdenum(pyridine)(hexamethylphosphoric triamide) (MoOPH), in the oxidation of enolate ions to α -hydroxycarbonyl compounds. It was hoped that, due to the steric bulk of this reagent, reaction with the anion derived from cotinine would provide largely *trans*-3'-hydroxycotinine. Indeed, reaction of cotinine with lithium diisopropylamide followed by treatment of the resulting anion with MoOPH provided an 80:20 mixture of trans/cis isomers of **5**, determined by capillary GC analysis. Unfortunately, we were not able to separate the desired trans isomer directly by crystallization, either as the free base or as a variety of salts. However, conversion to solid hexanoate ester **6** followed by recrystallization removed the cis isomer and unreacted cotinine present in the mixture. Heating the purified ester with *n*-butylamine provided **4** that was >98% trans isomer, $[\alpha]_D^{22} = +42.2^\circ$ (Scheme I). The melting point was in good agreement with values reported for the human,⁸ dog,⁸ and monkey metabolite.⁹ The optical rotation was substantially identical with the values reported for this metabolite isolated from human and dog urine. Proton NMR data further supported the stereochemical assignment. Dagne and Castagnoli reported that the signals for the C-4' methylene protons of metabolic *trans*-3-hydroxycotinine occur as overlapping multiplets centered at δ 2.5, whereas the corresponding methylene proton signals of synthetic *cis*-

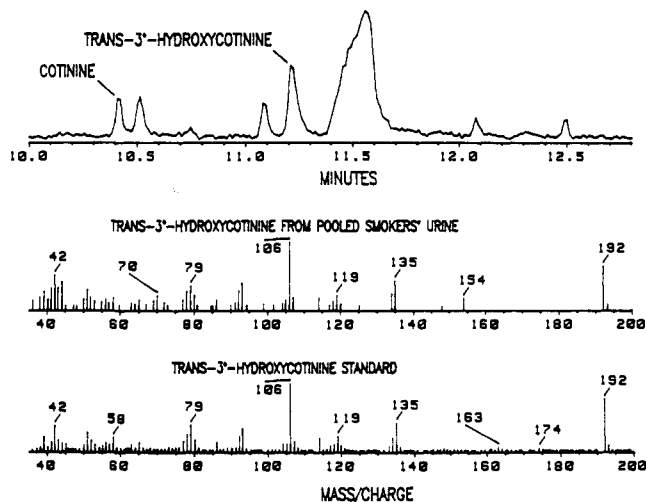


Figure 1. GC-MS analysis of a pooled smokers' urine sample. Upper panel, total ion chromatogram of urine extract; middle panel, electron-impact mass spectrum of 3'-hydroxycotinine in urine extract, scan at apex of peak with retention time of 11.22 min; lower panel, mass spectrum of synthetic *trans*-3'-hydroxycotinine.

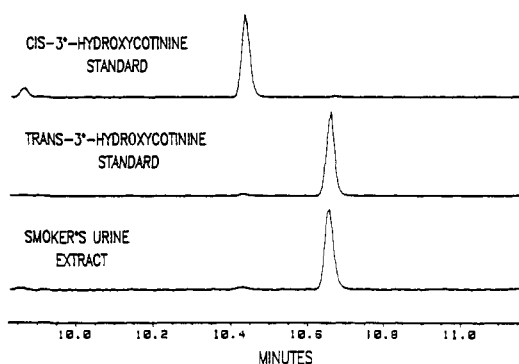


Figure 2. Determination of stereochemical purity of 3'-hydroxycotinine by selected ion monitoring GC-MS analysis of the chloro derivatives. Upper panel, m/z 210 ion chromatogram of *trans*-3'-chlorocotinine derived from synthetic (\pm)-*cis*-3'-hydroxycotinine; middle panel, m/z 210 ion chromatogram of the chloro derivative from synthetic (3'R,5'S)-*trans*-3'-hydroxycotinine; lower panel, m/z 210 ion chromatogram of the thionyl chloride derivatized extract of a smoker's urine sample.

3'-hydroxycotinine occur as separate multiplets, centered at δ 1.8 and 2.9.⁹ The 500-MHz NMR spectrum of our synthetic *trans*-3'-hydroxycotinine displayed a 16-line multiplet (two protons) centered at δ 2.51. In the 500-MHz NMR spectrum of *cis*-3'-hydroxycotinine, the signals for the methylene protons occurred as two distinct multiplets, at δ 1.83 (6 lines) and at δ 3.06 (8 lines).

Metabolic Studies

Hydroxycotinine was identified in an extract of pooled smokers' urine by GC-MS. The electron-impact mass spectrum was in good agreement with a mass spectrum obtained from synthetic *trans*-3'-hydroxycotinine (Figure 1).

To determine the stereochemical purity of synthetic and metabolic 3'-hydroxycotinine, samples were converted to 3'-chlorocotinine by reaction with thionyl chloride. This reaction (Scheme I) has been shown by NMR to give 3'-chlorocotinine (**7**) with inversion of stereochemistry.¹⁸ The

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Table I. Isomeric Composition of 3'-Hydroxycotinine Excreted in Urine of Smokers

subject	ratio trans/cis	subject	ratio trans/cis
SC	96:4	SM	96:4
TB	96:4	GW	98:2
PW	95:5	AS	95:5
SK	95:5		

chloro derivatives, in contrast to *cis*- and *trans*-3'-hydroxycotinine, have good chromatographic properties and are readily separated by capillary GC (Figure 2). In this way, we were able to determine that our synthetic *trans* isomer and the *cis* isomer prepared by the method of Dagne and Castagnoli⁹ were both >98% pure.

Extracts of urine from several smokers were derivatized with thionyl chloride and subjected to GC-MS analysis. Selected ion monitoring of *m/z* 210, the molecular ion of 3'-chlorocotinine, and integration of the reconstructed ion chromatograms indicated that hydroxylation of (*S*)-cotinine in humans is highly stereoselective (Figure 2). In seven subjects, the metabolite was 95–98% *trans* (Table I). Since some epimerization of the chloro derivatives could have occurred during derivatization or chromatography, our estimate is the minimum stereochemical purity of 3'-hydroxycotinine produced in humans.

There has been considerable interest among clinicians and epidemiologists in determining concentrations of nicotine and cotinine in biologic fluids, in order to estimate exposure to tobacco smoke.^{19–21} Since concentrations of 3'-hydroxycotinine generally exceed nicotine and cotinine concentrations in the urine of smokers,^{4,10} this metabolite would appear to be an excellent marker of nicotine intake. However, in order to obtain an estimate of exposure based on urinary or plasma concentrations, it will be necessary to determine the metabolic disposition of the metabolite in humans. Availability of (3'*R*,5'*S*)-3'-hydroxycotinine and synthetic methodology suitable for preparation of isotopically labeled derivatives will facilitate these studies.

Experimental Section

Melting points were taken on a Laboratory Devices Mel-Temp apparatus and are uncorrected. Mass spectral analyses were carried out by GC-MS with a Hewlett-Packard 5890 GC with a capillary direct interface to a quadrupole mass spectrometer, Hewlett-Packard 5970B. Mass spectra and ion chromatograms were obtained with a Hewlett-Packard 59970B MS Data System. Proton NMR spectra were recorded on a General Electric 500-MHz instrument linked to a Nicolet 1180 computer. Infrared spectra were obtained from a Beckman Instrument Acculab grating spectrophotometer, and were done with solid samples milled in mineral oil.

Mixed Isomers of 3'-Hydroxy-(5'*S*)-cotinine (5). A solution of 5.6 mL (4.04 g, 40 mmol) of diisopropylamine in 35 mL of dry THF (dried by distillation from sodium benzophenone ketyl) was stirred magnetically in a round-bottom flask under argon. This solution was cooled to -78 °C with an external dry ice/acetone bath, and 25 mL of a 1.6 M solution of *n*-butyllithium in hexane (40 mmol) was added. The yellow solution was brought up to 0 °C, held there for a few minutes with continuous stirring, and then recooled to -78 °C. There was then added a solution of 3.52 g of (*S*)-cotinine (20 mmol, commercially available from Aldrich Chem. Co. or prepared from (*S*)-nicotine²²) in 20 mL of dry THF.

A heavy, yellow crystalline mass developed which was broken up by raising the temperature to 0 °C. The stirred suspension was again cooled to -78 °C and, against a strong counterflow of argon, 17.36 g of MoOPH (40 mmol)¹⁶ was added with occasional swirling. The quite dark reaction mixture was brought to 0 °C for 15 min, and then to about 10 °C for an additional 15 min with stirring. The reaction was quenched by the addition of 80 mL of a saturated aqueous solution of sodium sulfite. The organic phase was separated, and the remaining aqueous phase and black interface were extracted first with 3 × 75 mL of a 50:50 mixture of methylene chloride and 2-propanol, followed by 50 mL of 2-propanol. The extracts were all pooled, and the solvents were removed on the rotary evaporator to yield 16.5 g of a deep blue residue. This was dried by azeotropic distillation of first 400 mL of methylene chloride and then 400 mL of chloroform using a rotary evaporator. The residual material was shown by GC analysis (see below) to be an 80:20 *trans/cis* mixture of 3'-hydroxy-(5'*S*)-cotinine, but all efforts to obtain a crystalline product failed.

(3'*R*,5'*S*)-*trans*-3'-Hydroxycotininyl Hexanoate (6). Crude 5 was dissolved in 100 mL of hexanoic anhydride, treated with 20 mL of pyridine, and held at steam bath temperature for 6 h. The deep blue color became brown almost immediately. The reaction mixture was quenched in 1 L of 1 M sulfuric acid, which produced a heavy, blue-black sludge. This was removed by filtration through paper in a Büchner funnel, yielding a clear, pale yellow-pink aqueous phase. This was extracted with 2 × 100 mL of methyl *tert*-butyl ether (MTBE). The aqueous phase was brought to neutrality with concentrated ammonium hydroxide which deposited a fine, silky, blue-black solid. This was removed by filtration through paper. The aqueous filtrate was then brought to approximately pH 9 with ammonium hydroxide and extracted with 3 × 100 mL of MTBE. Removal of the solvent yielded 6.3 g of a clear salmon oil that contained mainly the *trans* isomer of the hexanoate ester of 3'-hydroxycotinine mixed with hexamethylphosphoric triamide derived from the oxidizing agent, with small but detectable quantities of the *cis* isomer and cotinine. This assay was performed by TLC on silica plates, using as developing solvent a mixture of ethyl acetate, methanol, and concentrated ammonium hydroxide in the ratio of 17:2:0.2. The *R_f*'s were 0.58, 0.50, and 0.35 for the *trans* isomer hexanoate, the *cis* isomer hexanoate, and cotinine, respectively. Further extraction of the aqueous phase with 2 × 100 mL of methylene chloride yielded, after removal of the solvent, a colorless oil that was largely cotinine by TLC.

The 6.3 g of crude ester was chromatographed on an 8.5 cm × 6 cm silica column using acetonitrile containing 2.5% ammonia as the eluting solvent. The eluates were assayed by TLC, and the fractions containing hydroxycotininyl hexanoate were combined. Removal of the solvent afforded 1.12 g crude *trans*-3'-hydroxycotininyl hexanoate as slightly gummy white crystals. This product was ground under 3 mL of cyclohexane, which was decanted from the mass. The remaining solids were then recrystallized from 20 mL of boiling cyclohexane to yield 0.79 g of the ester which had only a slight trace of the *cis*-ester by TLC. A second recrystallization from 7.9 mL of cyclohexane (with filtration of the hot solution through a heated, fine-sintered-glass disk to remove a small amount of turbidity) yielded 0.66 g of *trans*-3'-hydroxycotininyl hexanoate, 12% yield from cotinine, as a fine, off-white product with no detectable *cis* isomer by TLC: the melting point was 71–72 °C with prior sintering at 70 °C; IR 1746 cm⁻¹ (ester C=O), 1696 (amide C=O) 1172, 1120, 1003, 821, 794, and 727. Anal. C₁₆H₂₂N₂O₃ requires: C, 66.18; H, 7.64; N 9.65. Found: C, 66.13; H, 7.70; N, 9.14.

Racemic *cis*-3'-hydroxycotinine, synthesized by the method of Dagne and Castagnoli,⁹ formed a hexanoate which melted at 61–64 °C from cyclohexane; IR 1741 cm⁻¹ (ester C=O), 1703 (amide C=O), 1186, 1121, 1032, 858, 821, and 723.

***trans*-(3'*R*,5'*S*)-3'-Hydroxycotinine Perchlorate (4).** The purified ester above (6) was dissolved in 6.5 mL of *n*-butylamine and held at reflux for 1.5 h, and the solvent was removed on a rotary evaporator. The last vestiges of the amine were removed under a hard vacuum at 0.2 mmHg. The residue (0.83 g) was dissolved in 10 mL of 2-propanol and with continuous stirring

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there was added 15 drops of 60% perchloric acid. The white solids that formed were removed by filtration, washed with 3 mL of 2-propanol, and finally with 10 mL MTBE. After air-drying to constant weight, there was obtained 0.56 g of (3'R,5'S)-3'-hydroxycotinine perchlorate as fine, white crystals with a melting point of 209–210 °C. The stereochemical purity, determined as described below, was >98% *trans*. A second crop from the mother liquors weighed 0.046 g and was off-white in color: mp 202–205 °C; yield 89%; ¹H NMR (D₂O) δ 2.51 [m, 16 lines, 2 H, (C4')-H₂], 2.82 (s, 3 H, NCH₃), 4.68 [t, 1 H, (C3')-H], 5.13 [dd, 1 H, (C5')-H], 8.11 [dd, 1 H, *J*₁ = 8 Hz, *J*₂ = 6 Hz, (C5)-H], 8.50 [dt, 1 H, (C4)-H], 8.75 [d, 1 H, *J* < 2 Hz, (C2)-H], 8.79 [d, 1 H, *J* = 6 Hz, (C6)-H] [lit.⁹ ¹H NMR δ 2.5 (m), 2.83 (s), 4.7 (m)]. Anal. C₁₀H₁₃ClN₂O₈ requires C, 41.04; H, 4.48; N, 9.57. Found: C, 40.83; H, 4.44; N, 9.52.

Racemic *cis*-3'-hydroxycotinine⁹ displayed the following ¹H NMR spectrum as the perchlorate in D₂O: δ 1.83 [m, 6 lines, 1 H, (C4')-H (*cis* to pyridyl)], 2.68 (s, 3 H, NCH₃), 3.06 [m, 8 lines, 1 H, (C4')-H (*trans* to pyridyl)], 4.85 [t, 1 H, (C3')-H], 4.92 [t, 1 H, (C5')-H], 8.12 [dd, 1 H, *J*₁ = 9 Hz, *J*₂ = 6 Hz, (C5)-H], 8.58 [dt, 1 H, (C4)-H], 8.79 [d, 1 H, *J* = 6 Hz, (C6)-H], 8.83 [d, 1 H, *J* < 2 Hz, (C2)-H] [lit.⁹ ¹H NMR δ 2.0 (m), 2.64 (s), 2.9 (m), 4.5 (m)].

A 100-mg portion of the perchlorate salt of 4 was converted to the base with 2 mL of saturated aqueous potassium carbonate and extracted with three 2-mL portions of 50:50 methylene chloride/isopropyl alcohol. The combined extracts were evaporated and chromatographed on a 2 cm × 1 cm silica column using 10:1:0.2 acetonitrile/methanol/concentrated ammonia as the eluting solvent. Those fractions containing 4 were evaporated and dried at 0.1 mmHg for 30 min to give a colorless, viscous oil. Scratching with a glass rod induced crystallization to a white solid: [α]_D²² = +42.2° (*c* 2.5, methanol); mp 107–108 °C after recrystallization from acetone; IR 3450 cm⁻¹ and 3230 (OH), 1670 (C=O), 1113, 1080, 1031, 960, 798, and 745. Metabolic *trans*-3'-hydroxycotinine isolated from urine of human subjects following administration of (*S*)-cotinine had [α]_D²⁹ = +42.5°, mp 110–112 °C.⁸ The melting point of 3'-hydroxycotinine isolated from the urine of a rhesus monkey was 110–111 °C whereas synthetic (±)-*trans*-3'-hydroxycotinine melted at 71–72 °C.⁹ The racemic *cis*-3'-hydroxycotinine⁹ had an IR spectrum with OH at 3100 cm⁻¹, 1693 (C=O), 1141, 1085, 1051, 1030, 848, 816, and 720.

Extraction and GC-MS Analysis of 3'-Hydroxycotinine from Smokers' Urine. To 1 mL of urine was added 1 mL of saturated aqueous potassium carbonate, and the mixture was extracted with 2.5 mL of 50:50 methylene chloride/isopropyl alcohol by vortex-mixing. The organic (upper) layer was separated and evaporated with a current of nitrogen with gentle heating (~50 °C). The residue was reconstituted in 100 μL of 90:10 toluene/1-butanol and analyzed by GC-MS on a 12 m × 0.2 mm

fused silica capillary column with an 0.33 μm cross-linked methylsilicone stationary phase. The injection was made in the splitless mode, with an injection-port temperature of 250 °C. Following a 1-min hold, the column oven temperature was programmed from 70 to 225 °C at 20 °C/min. The mass spectral analysis was carried out by using electron-impact ionization at 70 eV and scanning from 35 to 210 amu (Figure 1).

GC-MS Analysis of 3'-Chlorocotinine Derived from 3'-Hydroxycotinine. Determination of Stereochemical Purity. To about 50 μg of *cis*- or *trans*-3'-hydroxycotinine or evaporated extract of urine (extracted as described above) in a culture tube was added 1 mL of methylene chloride, 5 drops of pyridine, and 1 drop of thionyl chloride. The tube was vortex-mixed briefly, and then the solvent was evaporated with a current of nitrogen. To the residue was added 0.5 mL of 1 M sulfuric acid and 2.5 mL of 70:30 toluene/1-butanol. The tube was vortex-mixed and centrifuged, and the organic layer was removed and discarded. Aqueous potassium carbonate (0.5 mL of 50%) was added, and the mixture was extracted with 100 μL of 90:10 toluene/1-butanol. A 1-μL aliquot of the extract was analyzed by GC-MS. The analysis was carried out using a 12 m × 0.2 mm fused silica capillary column with a cross-linked 5% phenylmethylsilicone stationary phase of 0.33 μm phase thickness. The injection was made in the splitless mode, with an injection-port temperature of 250 °C. Following a 1-min hold, the temperature was programmed from 70 to 225 °C at 20 °C/min. The mass spectrometer was operated in the selected ion monitoring mode with the molecular ion, *m/z* 210, and a major fragment, *m/z* 118, monitored. The *m/z* 210 ion chromatograms were integrated and peak areas of the *trans*- and *cis*-3'-hydroxycotinine-derived chlorocotinites were used to determine isomeric purity. The ratio of peak areas was adjusted to correct for a slight difference in extraction, derivatization, and/or ionization efficiency of the *cis* and *trans* isomers. The peak area ratio of *cis/trans*-3'-hydroxycotinine chloro derivatives from a nonsmoker's urine specimen spiked with equal amounts of each isomer was 1.32. The retention time of *cis*-3'-chlorocotinine (derived from *trans*-3'-hydroxycotinine) was 10.66 min; the retention time of *trans*-3'-chlorocotinine (derived from *cis*-3'-hydroxycotinine) was 10.43 min (Figure 2).

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Registry No. 1, 54-11-5; 3, 486-56-6; 4, 34834-67-8; 4·HClO₄, 126874-95-1; *cis*-5, 37096-14-3; 6, 126874-93-9; *cis*-6, 126874-94-0; 7, 126874-96-2; *trans*-7, 126874-97-3.