ANTIMETABOLITES PRODUCED BY MICROORGANISMS. XIV[1]

2-METHYL-L-ARGININE, A NEW AMINO ACID WITH ANTIBIOTIC PROPERTIES

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A new arginine antimetabolite was isolated from the fermentation broth of a new strain of *Streptomyces* and identified as 2-methyl-L-arginine.

A number of antimicrobially active phytogenic and microbiogenic arginine derivatives, whose activities are reversed by arginine, have been described recently.[2,3,4] We now report a new member of this series, 2-methyl-L-arginine (**1**), produced by a new *Streptomyces* species designated X-11837.

Taxonomy of Species X-11837

Microscopic Characteristics

Culture X-11837, isolated from farmyard soil collected in Port Harcourt, Nigeria, grew on agar media with aerial hyphae and well developed branched mycelium penetrating into the agar. The submerged mycelium did not fragment, whereas some of the aerial hyphae differentiated into spore chains. The chains are spiral with more than 10 spores per chain. The spores are spiny (Figs. 1 and 2), with average measurements of 0.5~1.0 μm by 1.3~1.7 μm. Chromatographic analysis of whole-cell hydrolyzate revealed the presence of diaminopimelic acid different from the meso-form.[5] According to these characteristics species X-11837 belongs to the genus *Streptomyces*.[5]

Macroscopic Characteristics

In Table 1, spore-mass color, color of substrate mycelium (underside), presence of soluble

Fig. 1. X-11837. Spore chains. 14 days on ISP 3 agar. ×1,335

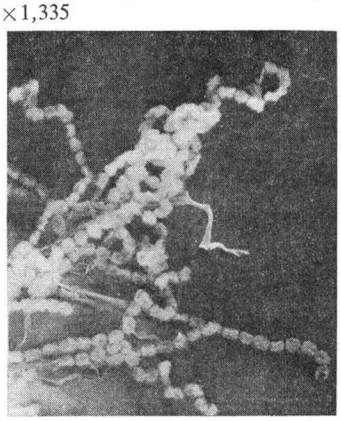


Fig. 2. X-11837. Spore chain. 14 days on ISP 3 agar. ×4,535

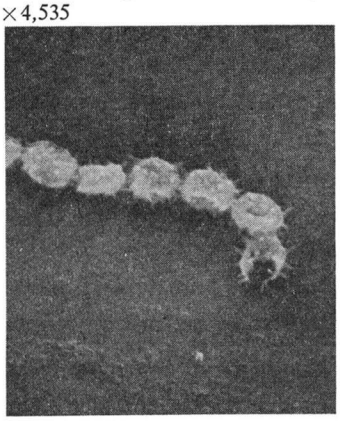


Table 1. Cultural characteristics of *Streptomyces* sp. X-11837

Medium	Amount of growth degree of sporulation	Spore-mass color	Color of substrate (underside) mycelium	Presence of soluble pigment
Bacto yeast-malt-extract agar (ISP2)	abundant growth, well sporulated, liquid exudate	2*ih* (dark covert gray) in center; 3*ca* (pearl pink) toward edge; *b* (oyster white) at edge	3*lg* (adobe brown); 3*gc* (light tan) at edges; patches of 3*nl* (dark brown)	—
Bacto oatmeal agar (ISP3)	moderate growth, moderate to well sporulated	3*li* (beaver) in center, a few flecks of *c* (light gray) at edge	3*ig* (beige brown) under sporulated part; 2*gc* (bamboo) at edge	—
Bacto inorganic salts starch agar (ISP4)	poor growth, little sporulation	*b* (oyster white) where sporulated	between 3*ie* (camel) and 3*gc* (beige)	—
Bacto glycerol-asparagine agar base plus 1.0% glycerol (ISP5)	moderate growth, moderate sporulation	3*ig* (beige brown) mostly, 2*dc* (natural) at edge	2*fe* (covert gray) where sporulated, 2*dc* (natural) at edges where not sporulated	—
Bacto peptone yeast-extract-iron agar (ISP6)	moderate growth, moderate sporulation	*b* (oyster white) in center		brown
Bacto tyrosine agar (ISP7)	abundant growth, well sporulated liquid exudate	3*ig* (beige brown) center, 2*dc* (natural) at edge		diffuse pink on plate, but not concentrated around colonies

pigment and amount of growth and sporulation are given for growth on six culture media[7] (Difco ISP2~ISP7). These data were recorded after 14 days of growth at 28°C; colors are described according to the Color Harmony Manual, 4th edition, 1958 (Container Corp. of America).

Physiological Characteristics

The organism utilized D-glucose, D-fructose, D-galactose, L-arabinose, D-xylose, sucrose, raffinose and D-mannitol, grew slightly or not at all with salicin, did not grow with L-rhamnose, *myo*-inositol, or cellulose; and hydrolyzed starch, gelatin, and casein.

Growth was observed in a liquid medium of Bacto ISP1 (tryptone yeast extract broth, Difco) at 10°, 28°, 37° and 45°C but was poor at 45°C. No growth occurred at 50°C. Growth was accompanied by darkening of the medium at 28° and 37°C. The organism grew on agar plates of Difco ISP2 and ISP3 media at 28°, 37° and 45°C.

The culture produced hydrogen sulfide (darkening in medium ISP6) but not melanin (no dark color in medium ISP7). Growth was observed in liquid medium[8] with sodium-chloride concentrations of up to 5%.

Streptomyces X-11837 was sensitive to 10 μg of streptomycin present on a 6-mm paper-disc.

Comparison with Known *Streptomyces* Species

Based on color of the spore mass, shape of the spore chain, spore surface and melanoid-pigment production as primary characters for species-group allocation, none of the reported

Streptomyces species[9,10] fit the description of culture X-11837. Among the *Streptomyces* species of the "gray" series with spiral spore-chains and spiny spores, only three species, *S. durhamensis*,[11] *S. filipinensis*[12] and *S. griseochromogenes*[11] resemble species X-11837 in the assimilation of most carbon sources, with the exception of *myo*-inositol utilization and melanoid-pigment production on ISP 7 agar. Their spore-mass colors on ISP media[7] were mainly in the white to beige color-series, whereas those of species X-11837 were predominantly in the beige-brown series. In contrast to species X-11837, *S. filipinensis* and *S. griseochromogenes* were slightly less tolerant to sodium chloride in the medium and *S. durhamensis* had spiral spore chains more tightly coiled than species X-11837. Further, production of **1** was not observed with any of the known strains under the fermentation conditions described below.

Production, Isolation and Identification of 2-Methyl-L-Arginine

Although *Streptomyces* sp. X-11837 elaborated **1** in a variety of media, those containing complex constituents such as soybean-meal hydrolyzate, yeast extract and others were superior to minimal media.

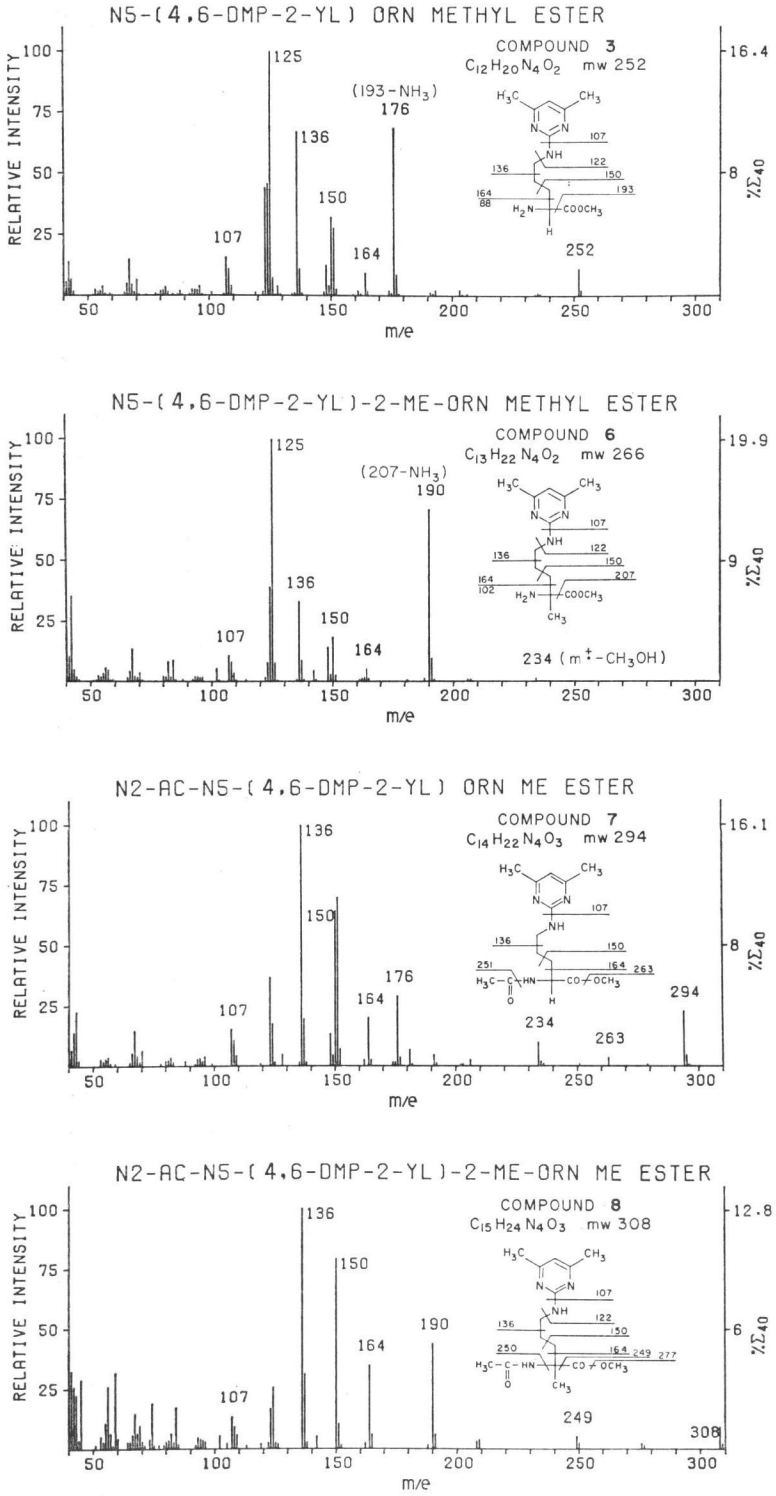
The organism was maintained on tomato-soy agar slants composed of (g/liter) tomato paste (Contadina), 20; glucose (Cerelose, Corn Products), 10; defatted soybean flour (Soyalose, Central Soya), 10; $CaCO_3$, 2; Bacto-peptone (Difco), 1; K_2HPO_4, 1; and agar, 20. Portions of slant growth were added to 6-liter Erlenmeyer flasks containing 2 liters of inoculum medium composed of (g/liter): tomato pomace (Seaboard Supply), 5; dried distiller's solubles (Soludri, Schenley), 5; cottonseed flour (Proflo, Trader's) 5; protopeptone #366 (Wilson), 5; cornstarch (Anheuser Busch), 10; $CaCO_3$, 1; and K_2HPO_4, 1. The flasks were incubated at 28°C for 72 hours on a rotary shaker (250 rpm, 5.08 cm throw). Four liters of the resulting inoculum were added to 225 liters of fermentation medium composed of (g/liter) glucose (Cerelose, Corn Products), 10; Bacto-peptone (Difco), 5; yeast extract (Difco), 3; and $FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$, 0.03. The culture was incubated at 28°C in a 380-liter fermentor, aerated at 0.15 m^3/min and agitated at 280 rpm. Silicone antifoam (Dow Corning AF) was added as needed to control frothing. After 24 hours the fermentation broth was clarified by centrifugation through infusorial earth.

The clarified broth was passed through a column of 20.5 cm diameter containing 50 liters of Dowex 50W-X4 (H^+) and the column was washed consecutively with 200 liters of water, 200 liters of 5% aqueous pyridine and 50 liters of water. The active fraction was then eluted with 1 N ammonium hydroxide solution and was mostly contained in the first 300 liters of ammoniacal effluent which, upon concentration and freeze-drying, gave crude **1** (55 g, approx. 1% purity).

To achieve additional purification the crude was dissolved in water, the pH of the solution adjusted to 3.5 with dil. hydrochloric acid, filtered and chromatographed on a column of Dowex 50W-X8 (Na^+), 200~400 mesh, 68×650 mm. The column was developed with a buffer (8 liters) prepared by adding a 0.1 M citric-acid solution to a 0.2 M dibasic sodium phosphate solution to pH 6.1. Development was continued with 32 liters of the same buffer but containing 5.84 g of sodium chloride per liter. The antimetabolite was eluted by the sodium-chloride containing buffer and started to emerge in the effluent after 17 liters had passed through the column.

The antimetabolite-containing effluent was desalted by passage through a column containing

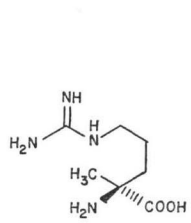
Fig. 3. Mass spectra of various N^5-(4,6-dimethylpyrimidin-2-yl)-ornithine methyl ester derivatives



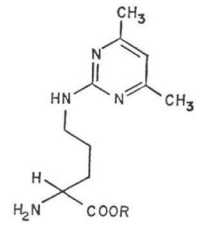
Dowex 50W-X4 (H^+), 50~100 mesh (2.5 liters) followed by a water wash and elution with 1 N ammonium hydroxide. The biologically active fractions were brought to dryness to yield crude **1** (2.6 g, approx. 20 % purity).

This preparation was chromatographed on a column (25×280 mm) of silica gel (Woelm, 0.05~0.2 mm) prepared by slurrying in chloroform and developed with system B. Most impurities preceded the biologically active fractions, the latter were concentrated to dryness under reduced pressure to yield an amorphous preparation (0.7 g) containing **1** and arginine in an approximate ratio of 2:1.

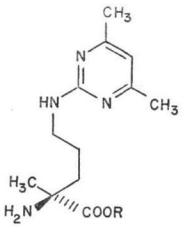
Pure **1** was isolated by chromatography of the arginine-containing preparation (200 mg) on a cellulose column, 40×215 mm (Cellex MX, Bio-Rad) with system A. 2-Methylarginine was eluted first and was quantitatively separated from arginine; concentration of the appropriate fractions gave **1** (133 mg) as amorphous powder. *Anal.* $C_7H_{16}N_4O_2 \cdot 2H_2O$ (224.26), C, H, N; ORD (*c* 0.224, 0.25 N HCl) $[\Phi]_{700}+14°$, $[\Phi]_{589}+21°$, $[\Phi]_{222}+690°$ (PK); $\delta^{D_2O}_{ext.\ TMS}$ 1.98 (s, CH_3-C), 1.78~2.54 (m, broad, CH_2-CH_2) and 3.75 (m, CH_2-N).



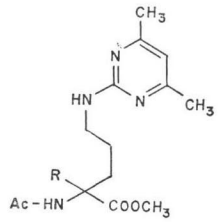
1



2 : R = H
3 : R = CH_3
4 : R = C_2H_5



5 : R = H
6 : R = CH_3



7 : R = H
8 : R = CH_3

The amorphous **1** base gave positive SAKAGUCHI and ninhydrin tests and **1** hydrochloride (tlc: Rf=0.15, Syst. A; 0.36, Syst. B; 0.11, Syst. C) exhibited chromatographic behavior similar to that of arginine hydrochloride.[3] The ^{1}H-nmr spectrum of **1** hydrochloride, $\delta^{D_2O}_{ext\ TMS}$ 1.93 (s, CH_3-C), 2.20 (broad m, CH_2-CH_2) and 3.65 (m, CH_2-N) lacked the signal for an α-hydrogen, expected at ~δ 4.22.[3] A spectral comparison with 2-methylalanine hydrochloride, $\delta^{D_2O}_{ext.\ TMS}$ 2.17 (s, CH_3-C-CH_3), suggested **1** to be 2-methylarginine, consistent with the observation of a ninhydrin-color intensity of **1** far weaker than that obtained with common amino acids.

To prove the structure, **1** was condensed with 2, 4-pentanedione to give **5** which was converted to methyl ester **6**, exhibiting a mass spectrum reminiscent of N^5-(4, 6-dimethylpyrimidin-2-yl) ornithine ethyl ester (**4**).[13] Although **6** did not give rise to a molecular ion, a direct mass spectral comparison (Fig. 3) between **6** and the corresponding methyl ester **3**, derived from arginine *via* **2**, revealed the similarity between the two compounds; most significantly, the spectrum of **6** contained a strong peak *m/e* 190, analogous to *m/e* 176 in the spectra of **3** and **4**, representing the loss of ammonia from the amine-fragment R-C(CH_3)=$\overset{+}{N}H_2$.[13] The mass spectrum of compound **8**, prepared by N-acetylation of **6**, exhibited the molecular ion, a spectral comparison with the corresponding arginine derivative **7** is shown in Fig. 3, confirming **8** as the 2-methyl derivative of **7** and establishing the structure of **1** as 2-methylarginine.

The assignment of chirality was based on a comparison of ORD spectra of L-arginine and **1**, both compounds reveal positive carboxyl n-π^* COTTON effects at *ca* 220 nm,[14] indicative of the L-configuration in **1**, **5**, **6** and **8**.

Antimicrobial Activity

The paper-disc agar-diffusion assay with *Escherichia coli* B as test organism in the minimal agar medium previously described[15] was employed for the initial detection and during isolation of the antimetabolite. Similar to N^5-hydroxy-L-arginine, the diameter of the inhibition zone was proportional to the logarithm of the concentration of **1** within the range of 2~100 μg/ml. A two-fold increase of the concentration of **1** increased the inhibition zone diameter by 3 mm and a 40 μg/ml solution gave a zone of about 36 mm; **1** showed approximately the same level of activity against *Bacillus* sp. ATCC 27860 as against *E. coli* B. Against *Bacillus subtilis* NRRL 558 **1** was 100-times less active and no activity could be demonstrated against *Serratia* sp. 101, *Klebsiella* sp. ATCC 27858, or *Candida albicans* NRRL 477 when tested in minimal medium.

The antibiotic activity of **1** against *Escherichia coli* was reversed by L-arginine, L-citrulline, L-ornithine and N^2-acetyl-L-ornithine but not by L-glutamic acid and N^2-acetyl-L-glutamic acid. The same reversal pattern was observed for N^5-hydroxy-L-arginine[16,17] suggesting similar biochemical involvement in effecting the antimicrobial activity.

Derivatives

Melting points were observed on a Reichert Thermopan hot stage and are uncorrected. Elemental analyses were within $\pm 0.4\,\%$ of calculated values. ^{1}Hnmr spectra were recorded on a Varian HA-100 spectrometer and mass spectra were obtained on a Varian MAT Model CH5 spectrometer operating at 70 eV with ion-source temperature of 250°C. Tlc was carried out with silica gel G plates (E. Merck, Darmstadt) employing systems A (chloroform - methanol - conc. ammonium hydroxide soln., 2:2:1, v/v), B (chloroform - methanol - conc. ammonium hydroxide soln. - water, 1:4:2:1, v/v), C (abs. ethanol - water - conc. ammonium hydroxide soln., 49:49:2, v/v) and D (chloroform - methanol, 8:1, v/v). All evaporations were conducted under reduced pressure.

2-Methyl-L-arginine 2-nitroindanedione salt

A solution of **1** dihydrate (47 mg, 0.21 mmol) in water (1 ml) was adjusted to pH 5 with a 1 M methanolic solution of 2-nitroindanedione, filtered and concentrated to a volume of 0.5 ml. The crystalline deposit formed upon refrigeration was recrystallized twice from hot water, to yield yellow prisms (62 mg, 0.15 mmol) which were dried (25°C, 25 mm, 3 hours), mp 170~172°C; *Anal.* $C_7H_{16}N_4O_2 \cdot C_9H_5NO_4 \cdot 2\frac{1}{2}H_2O$ (424.41), C, H, N, H_2O; $[\alpha]_D$ +7.8 (*c* 0.3, water), further loss of water occurred readily on continued drying at elevated temperature.

N^5-(4, 6-Dimethylpyrimidin-2-yl)-2-methyl-L-ornithine (**5**)

A mixture of **1** dihydrate (56.6 mg, 0.253 mmol), sodium hydrogencarbonate (59 mg), water (0.6 ml), ethanol (1 ml) and 2, 4-pentanedione (1 ml) was kept in a sealed tube at 100°C for 5 hours. The mixture was concentrated to near dryness, diluted with 2 N acetic acid (2 ml), heated on the steam bath for 15 minutes and extracted twice with ether.[13] The aqueous phase was charged onto a column of Dowex 50W-X4 (H^+), the column was washed with water (60 ml) and eluted with a mixture of water and pyridine, 9:1, v/v (50 ml). Concentration of the pyridine-containing effluent gave **5** as amorphous solids (60 mg, 0.238 mmol), δ_{TMS}^{TFA} 1.86 (s, CH_3-C), 1.88~2.38 (m, CH_2-CH_2), 2.56 (s, 2 CH_3-C$\leqslant$), 3.73 (m, CH_2-N) and 6.76 (s, H-C$\leqslant$).

N^5-(4, 6-Dimethylpyrimidin-2-yl)-2-methyl-L-ornithine methyl ester (**6**) and N^5-(4, 6-dimethylpyrimidin-2-yl) ornithine methyl ester (**3**)

Methanol (5 ml) was cooled to 0°C and acetyl chloride (1 ml) was added dropwise under stirring. To this solution **5** (60 mg) was added in one portion and the mixture was refluxed

for 3 hour. The solution was concentrated to dryness, taken up in chloroform (8 ml) and saturated with ammonia gas (30 min).[13] The resulting suspension was dried ($MgSO_4$) and filtered, the filtrate was concentrated to a viscous residue which crystallized on standing. Two recrystallizations from ether-cyclohexane afforded pure **6** as prisms, mp 56~58°C; *Anal.* $C_{13}H_{22}N_4O_2$ (266.35), C, H, N; $[\alpha]_D$ +13.3° (*c* 1.0, ethanol); Rf 0.29 (system D); $\delta^{CDCl_3}_{TMS}$ 1.32 (s, CH_3-C), 1.45~1.90 (m, CH_2-CH_2, NH_2), 2.27 (s, 2 CH_3-C⩽), 3.39 (dt, $J_{NH,5}=J_{4,5}=6.5$ Hz), 3.68 (s, OCH_3), 5.02 (broad, NH) and 6.28 (s, HC⩽).

Methyl ester **3** was prepared in an analogous fashion from **2**,[13] recrystallization from chloroform-ether-petroleum ether gave needles, mp 65~66°C; *Anal.* $C_{12}H_{20}N_4O_2$ (252.32), C, H, N; Rf 0.28 (system D); $\delta^{CDCl_3}_{TMS}$ 1.52~1.97 (m, CH_2-CH_2, NH_2), 2.25 (s 2 CH_3-C⩽), 3.50 (m, broad, CH_2-N), 3.71 (s, CH_3O), 5.10 (broad, NH) and 6.29 (s, CH⩽).

N^2-Acetyl-N^5-(4, 6-dimethylpyridin-2-yl)-2-methyl-L-ornithine methyl ester (**8**) and N^2-acetyl-N^5-(4, 6-dimethylpyrimidin-2-yl) ornithine methyl ester (**7**)

Methyl ester **6** (10 mg) was dissolved in methanol (1 ml), acetic anhydride (0.2 ml) was added and the solution was kept at room temperature for 1 hour. The solvents were removed and the residue chromatographed on a plate (20×20×0.25 mm) with system D, yielding **8** as fine needles from ether, mp 106°C; Rf=0.55 (system D); $\delta^{CDCl_3}_{TMS}$ 1.25~2.0 (m, CH_2-CH_2), 1.40 (s, CH_3-C), 1.97 (s, CH_3CO), 2.27 (s, 2 CH_3-C⩽), 3.38 (dt, $J_{NH,5}=J_{4,5}=6.5$ Hz), 3.73 (s, CH_3O), 4.94 (broad, NH) and 6.30 (s, HC⩽, NHCO).

Methyl ester **7** was prepared similarly by dissolving **3** (50.5 mg, 0.20 mmol) in methanol (5 ml) and addition of acetic anhydride (1 ml). The mixture was concentrated to dryness after standing for one hour, redissolved in chloroform (15 ml), the chloroform was washed with sodium hydrogencarbonate solution and with water, dried ($MgSO_4$) and concentrated to near dryness. Addition of ether and cyclohexane afforded needles (53 mg, 0.18 mmol), mp 116°C after one recrystallization from chloroform-ether-cyclohexane; *Anal.* $C_{14}H_{22}N_4O_3$ (294.36), C, H, N; Rf 0.53 (system D); $\delta^{CDCl_3}_{TMS}$ 1.50~2.0 (m, CH_2-CH_2), 2.03 (s, CH_3-CO), 2.27 (s, 2 CH_3-C⩽), 3.43 (dt, $J_{NH,5}=J_{4,5}=6.5$ Hz), 3.72 (s, CH_3O), 4.62 (m, H-2), 6.28 (H-C⩽) and 6.5 (broad, 2 NH).

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