

Supplementary Material

Characterization of a CholesterONitrone (ISQ-201), a Novel Drug Candidate for the Treatment of Ischemic Stroke

Emma Martínez-Alonso ¹, Alejandro Escobar-Peso ¹, Maria I. Ayuso ², Rafael Gonzalo-Gobernado ², Mourad Chioua ³, Juan J. Montoya ⁴, Joan Montaner ^{2,5}, Israel Fernández ^{6,*}, José Marco-Contelles ³, and Alberto Alcázar ^{1,*}

¹ Department of Research, IRYCIS, Hospital Ramón y Cajal, Ctra. Colmenar km 9.1, Madrid 28034, Spain; emma.martinez@hrc.es (E.M.-A.), alejandro.escobar@hrc.es (A.E.-P.), alberto.alcazar@hrc.es (A.A.)

² Neurovascular Research Group, Institute of Biomedicine of Seville, IBiS, Hospital Universitario Virgen del Rocío, Av. Manuel Siurot s/n, Seville 41013, Spain; mayuso-ibis@us.es (M.I.A.), rgonzalo-ibis@us.es (R.G.-G.), jmontaner-ibis@us.es (J.M.)

³ Laboratory of Medicinal Chemistry (IQOG, CSIC), C/Juan de la Cierva 3, Madrid 28006, Spain; mchioua@gmail.com (M.C.), jmarco@iqog.csic.es (J.M.-C.)

⁴ Isquaemia Biotech SL, Scientific Technological Park, C/Astrónoma Cecilia Payne s/n, Córdoba 14014, Spain; jimontoya@canaanrd.com

⁵ Neurovascular Research Laboratory, Institut de Recerca Vall d'Hebron, Autònoma University of Barcelona, Barcelona 08035, Spain; joan.montaner@vhir.org

⁶ Departamento de Química Orgánica I y Centro de Innovación en Química Avanzada (ORFEO-CINQA), Complutense University of Madrid, Av. Complutense s/n, Madrid 28040 Spain; israel@quim.ucm.es

Contents

A. Synthesis of cholesteronitrones ISQ-201/ ISQ-202.....S2-S9

B. Synthesis of cholesteronitrones ISQ-201 /ISQ-202 from natural cholesterol-plant.....S10-S15

A. Synthesis of cholesteronitrones ISQ-201 /ISQ-202

1. General methods for chemistry synthesis

Reactions were monitored by TLC using precoated silica gel aluminium plates containing a fluorescent indicator (Merck, 5539). Detection was done by UV (254 nm) followed by charring with sulfuric-acetic acid spray, 1% aqueous potassium permanganate solution or 0.5% phosphomolybdic acid in 95% EtOH. Anhydrous Na_2SO_4 was used to dry organic solutions during work-ups and the removal of solvents was carried out under vacuum with a rotary evaporator. Flash column chromatography was performed using silica gel 60 (230-400 mesh, Merck). Melting points were determined on a Kofler block and are uncorrected. IR spectra were obtained on a Perkin-Elmer Spectrum One spectrophotometer. ^1H NMR spectra were recorded with a Varian VXR-200S spectrometer, using tetramethylsilane as internal standard and ^{13}C NMR spectra were recorded with a Bruker WP-200-SY. All the assignments for protons and carbons were in agreement with 2D COSY, HSQC, HMBC, and 1D NOESY spectra. The purity of compounds was checked by elemental analyses, conducted on a Carlo Erba EA 1108 apparatus, and confirmed to be $\geq 95\%$.

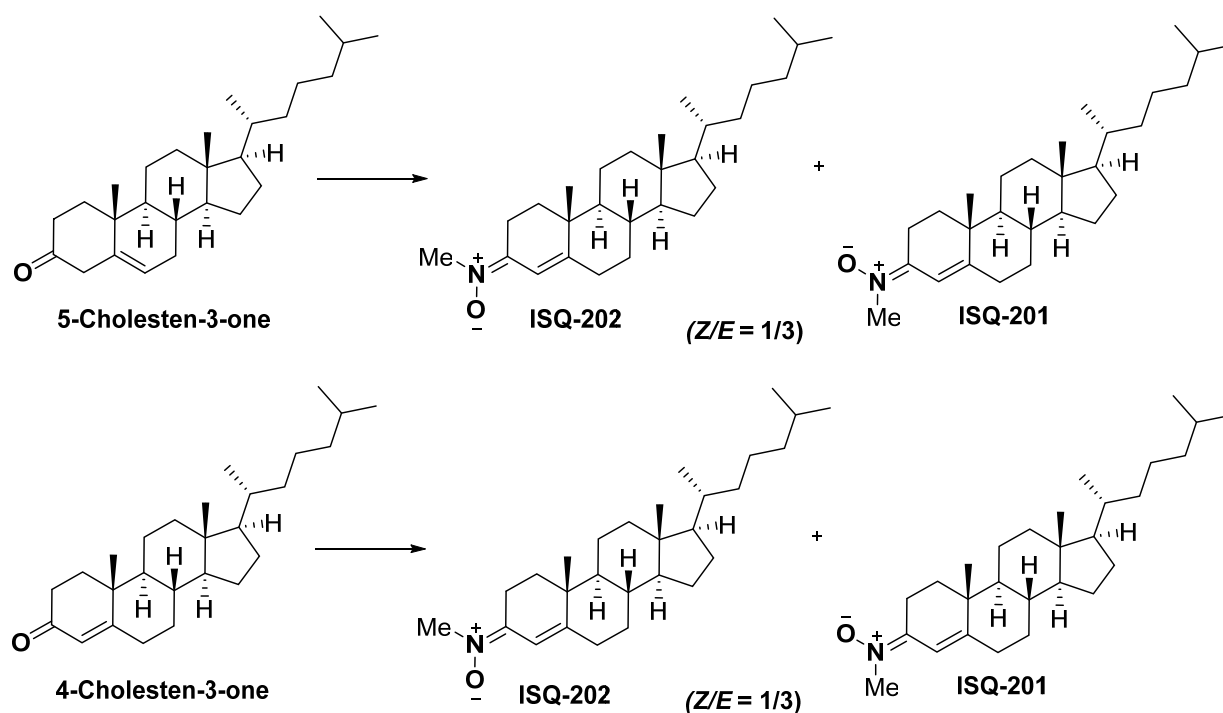
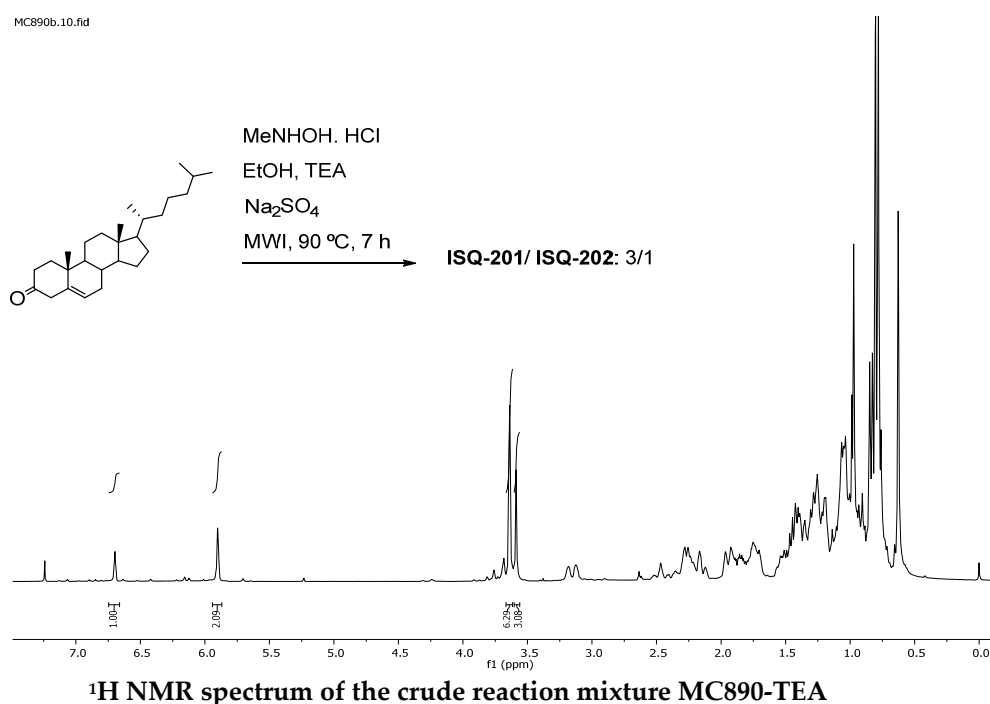


Figure 1. Synthesis of ChNs *E*-(ISQ-201), and *Z*-(ISQ-202) from 5-cholesten-3-one and 4-cholesten-3-one.

2. General procedure for cholesteronitrone (ChN) synthesis

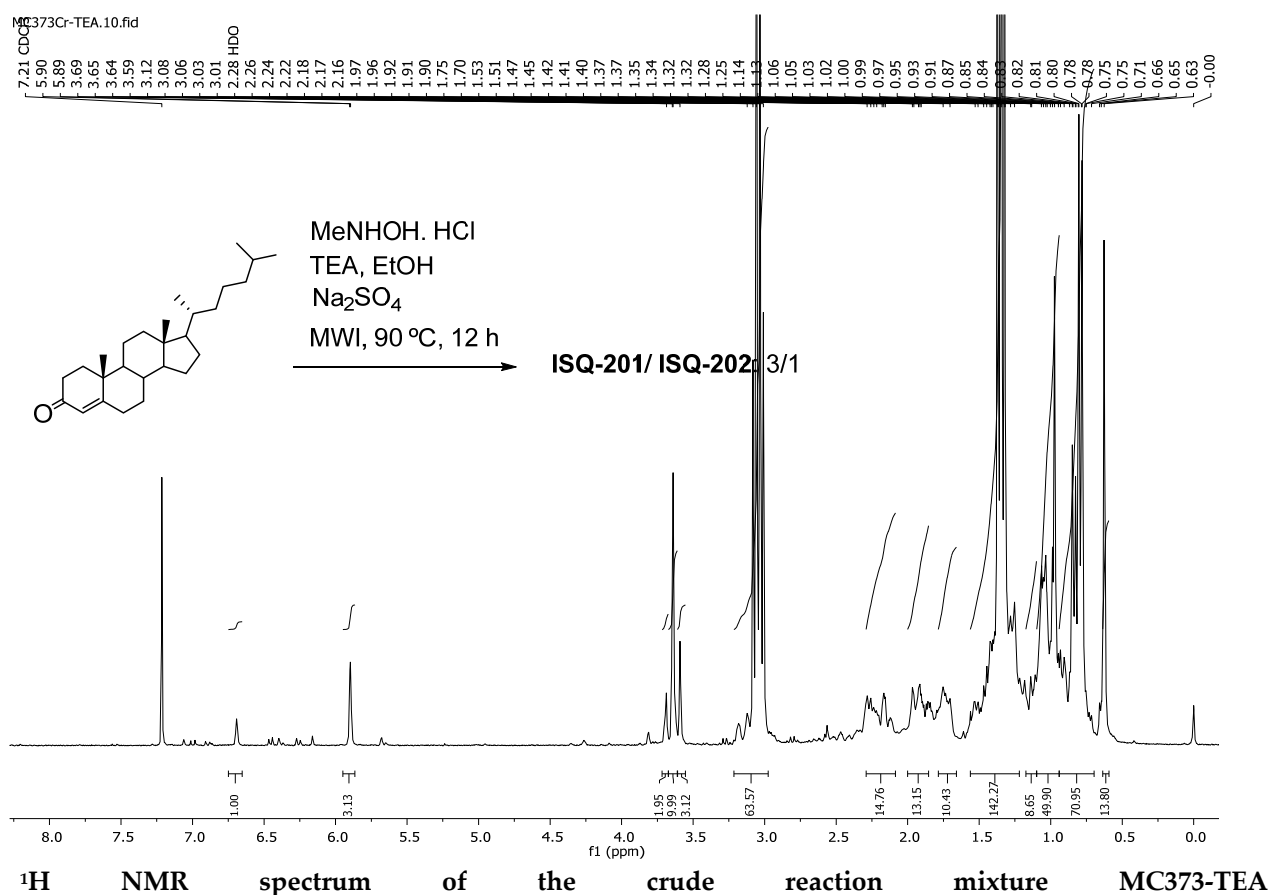
A solution of the ketone (1 mmol), dry Na₂SO₄ (3 mmol), and triethylamine (2 mmol) were suspended in EtOH. Then, the hydroxylamine hydrochloride (1.5 mmol) was added. The mixture was stirred for 30 s and then exposed to MWI (250 W) at 90 °C during the time indicated for each compound. When the reaction was over (tlc analysis), the solvent was removed under reduced pressure and diluted with water, extracted with AcOEt, dried over anhydrous sodium sulfate, filtered, and the solvent was evaporated. The resultant solid was purified by column chromatography (CH₂Cl₂/MeOH, from 1% to 3%) to give pure compounds *E*-(ISQ-201), and *Z*-(ISQ-202)¹ (Figure 1).

2.2. Synthesis of ChNs *E*-(ISQ-201), and *Z*-(ISQ-202) from 5-cholesten-3-one. Following the general procedure, reaction of commercial 5-cholesten-3-one (385 mg, 1 mmol), Na₂SO₄ (426 mg, 3 mmol), triethylamine (0.14 ml, 2 mmol), and methylhydroxylamine hydrochloride (126 mg, 1.5 mmol) in ethanol (10 mL), after 7 h, and column chromatography (CH₂Cl₂/MeOH, from 1% to 3%), gave a mixture of ISQ-201 and ISQ-202 in a 3:1 ratio, respectively, that after careful chromatography allowed us to obtain pure samples of ISQ-201 (296 mg, 71%), ISQ-201 + ISQ-202 (12 mg, 3%), and ISQ-202 (98 mg, 24%).

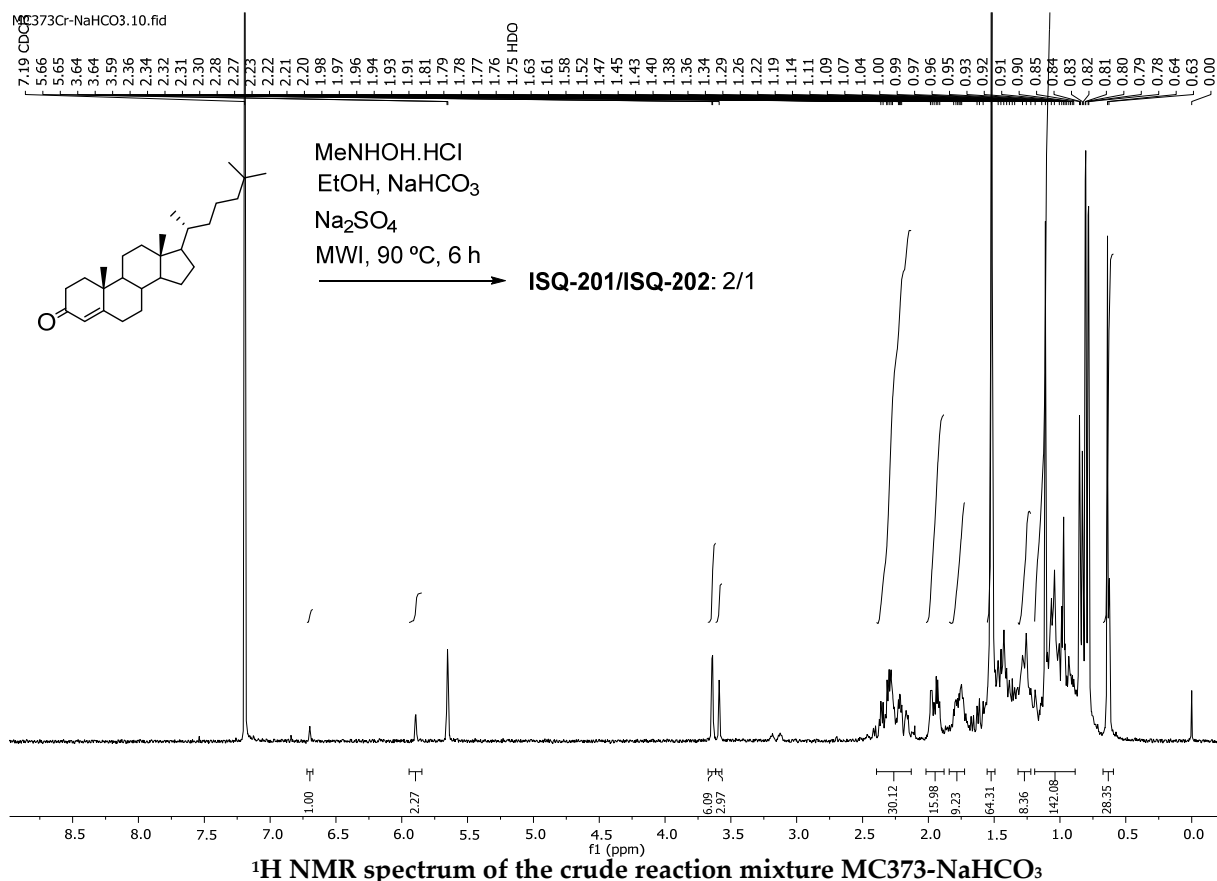


2.3. Synthesis of ChNs *E*-(ISQ-201), and *Z*-(ISQ-202) from 4-cholesten-3-one.

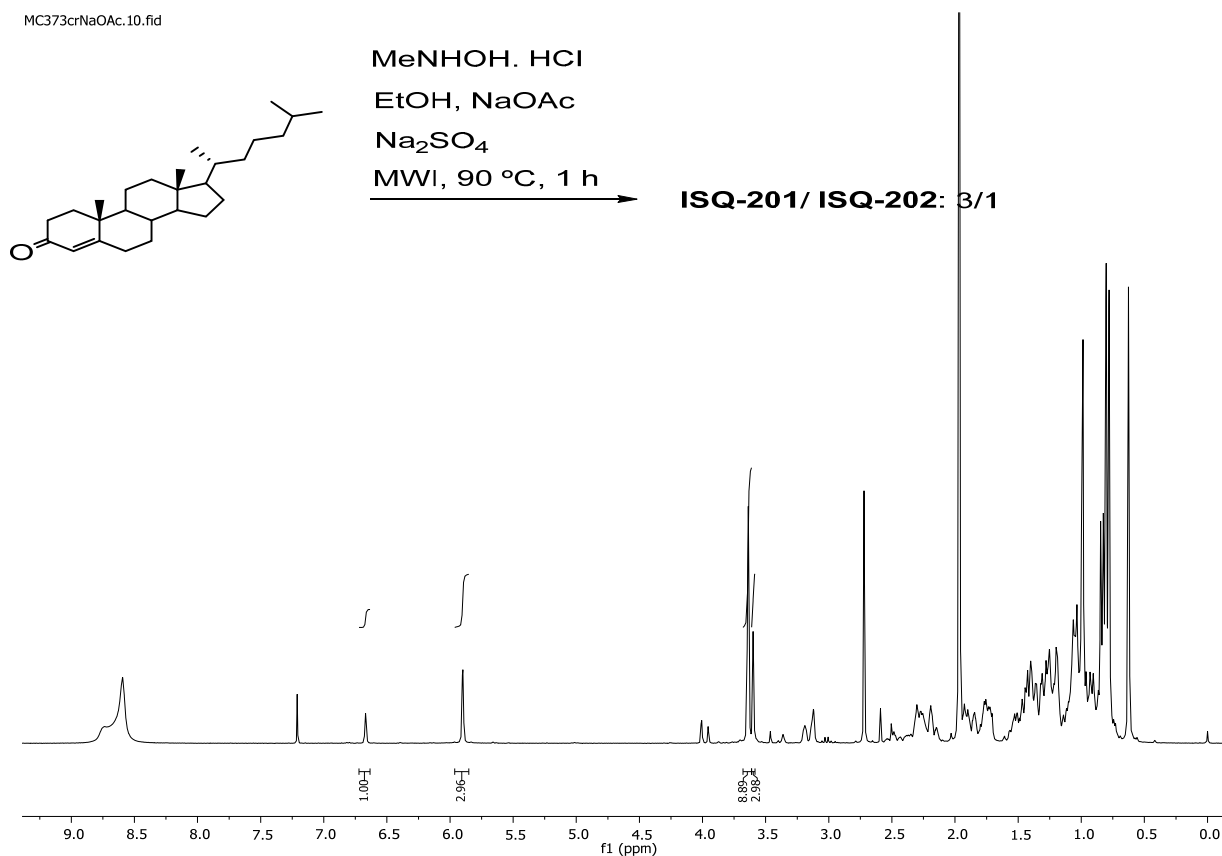
(A) Following the general procedure, reaction of commercial 4-cholesten-3-one (385 mg, 1 mmol), Na₂SO₄ (426 mg, 3 mmol), **triethylamine** (0.14 ml, 2 mmol), and methylhydroxylamine hydrochloride (126 mg, 1.5 mmol) in Ethanol (10 mL), after 12 h, gave a mixture of **ISQ-201** and **ISQ-202**, in a 3:1 ratio, that after careful chromatography (CH₂Cl₂/MeOH, from 1% to 3%), gave **ISQ-201** (273 mg, 66%), **ISQ-201 + ISQ-202** (12 mg, 3%), and **ISQ-202** (91 mg, 22%).



(B) Following the general procedure, reaction of commercial 4-cholesten-3-one (385 mg, 1 mmol), Na₂SO₄ (426 mg, 3 mmol), NaHCO₃ (168 mg, 2 mmol), and methylhydroxylamine hydrochloride (126 mg, 1.5 mmol) in ethanol (10 mL), after 7 h, gave a mixture of **ISQ-201** and **ISQ-202**, in a in a 2:1 ratio, that after careful chromatography (CH₂Cl₂/MeOH, from 1% to 3%), gave **ISQ-201** (205 mg, 49%) and **ISQ-202** (100 mg, 25%).



(C) Following the general procedure, reaction of commercial 4-cholesten-3-one (385 mg, 1 mmol), Na₂SO₄ (426 mg, 3 mmol), NaOAc (164 mg, 2 mmol), and methylhydroxylamine hydrochloride (126 mg, 1.5 mmol) in ethanol (10 mL), after 1 h, and column chromatography (CH₂Cl₂/MeOH, from 1% to 3%), gave a mixture of **ISQ-201** and **ISQ-202** in a 3:1 ratio. Repeated and careful chromatography (CH₂Cl₂/MeOH, from 1% to 3%), afforded **ISQ-201** (299 mg, 73%), **ISQ-201 +ISQ-202** (12 mg, 3%), and **ISQ-202** (98 mg, 23%).



¹H NMR spectrum of the crude reaction mixture MC373-AcONa

ISQ-201 : White solid; *R_f* (0.21, CH₂Cl₂/MeOH, 5%); mp 139-141 °C [*anti*(= *E*) isomer: mp 127-131 °C¹]; IR (KBr) ν max 2939, 2868, 2849, 1466, 1215 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 5.97 (d, *J* = 2.0 Hz, 1H, ⁴CH), 3.72 (s, 3H, NCH₃), 3.23 (d, *J* = 18.4 Hz, 1H, ²CH), 2.34 (m, 2H, ⁶CH₂), 2.21 (m, 1H, ²CH), 1.99 (m, 2H, CH₂), 1.80 (m, 2H, CH₂), 1.60 (s, 3H, CH₃), 1.36 (m, 10H, 5CH₂), 1.12 (m, 6H, 6CH₂), 1.04 (s, 3H, ¹⁹CH₃), 0.99 (m, 2H, CH₂), 0.91 (d, *J* = 6.4 Hz, 3H, ²¹CH₃), 0.88 (d, *J* = 1.3 Hz, 3H, ²⁶CH₃), 0.86 (m, 3H, ²⁷CH₃), 0.70 (s, 3H, ¹⁸CH₃); ¹³C NMR (101 MHz, CDCl₃) δ 156.8 (³C), 146.4 (⁵C), 112.9 (⁴CH), 56.1 (¹⁷CH), 55.9 (¹⁴CH), 53.5 (⁹CH), 46.0 (¹³C), 42.3 (NCH₃), 39.6 (C), 39.4 (C), 37.9 (¹⁰C), 36.1 (C), 35.77 (C), 35.73 (C), 34.4 (C), 33.4 (C), 32.2 (²⁵CH₂), 28.1 (¹⁶CH₂), 27.9 (²CH₂), 24.2 (¹⁵CH₂), 23.8 (²⁴CH₂), 22.7 (²⁶CH₃), 22.5 (²⁷CH₃), 21.4 (CH₂), 21.3 (¹¹CH), 18.6 (¹⁹CH₃), 17.8 (²¹CH₃), 11.9 (¹⁸CH₃). MS (EI) *m/z*: 413 (M, 37%), 398 (M-CH₃, 27%), 397 (M-O, 70), 137 (C₈H₁₁NO, 100%); MS (ESI) *m/z*: 414.2 (M + H)⁺, 436.2 (M + Na)⁺, 827.8 (2M)⁺, 849.7 (2M + Na)⁺.
 Anal. Calcd for C₂₈H₄₇NO: C, 81.29; H, 11.45; N, 3.39. Found: C, 80.98; H, 11.29; N, 3.44.

ISQ-202: White solid; *R_f* (0.20, CH₂Cl₂/MeOH, 5%); mp 153-155 °C [*syn*(= *E*) isomer: mp 146-149 °C]; IR (KBr) ν_{max} 2936, 2868, 1629, 1214 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 6.78 (s, 1H, ⁴CH), 3.66 (s, 3H, NCH₃), 2.44 (m, 4H, 2CH₂), 1.88 (m, 4H, 2CH₂), 1.37 (m, 14H, 7CH₂), 1.04 (s, 3H, ¹⁹CH₃), 0.98 (m, 2H, CH₂), 0.91 (d, *J* = 6.4 Hz, 3H, ²¹CH₃), 0.88 (d, *J* = 1.4 Hz, 3H, ²⁶CH₃), 0.85 (d, *J* = 1.4 Hz, 3H, ²⁷CH₃), 0.70 (s, 3H, ¹⁸CH₃); ¹³C NMR (101 MHz, CDCl₃) δ 123.7 (³C), 120.3 (⁵C), 113.7 (⁴CH), 56.0 (¹⁷CH), 55.9 (¹⁴CH), 53.5 (⁹CH), 46.4 (¹³C), 42.3 (NCH₃), 39.6 (C), 39.4 (C), 37.9 (¹⁰C), 36.0 (C), 35.72 (C), 35.71 (C), 35.4 (C), 32.9 (C), 32.2 (C), 28.1 (C), 27.9 (C), 24.1 (¹⁶CH₂), 23.7 (¹⁵CH₂), 23.6 (²⁴CH₂), 22.7 (²⁶CH₃), 22.5 (²⁷CH₃), 21.3 (¹¹CH₂), 18.6 (¹⁹CH₃), 17.8 (²¹CH₃), 11.9 (¹⁸CH₃). MS (EI) *m/z*: 413 (M, 37%)⁺, 398 (M-CH₃, 27%), 397 (M-O, 70), 137 (C₈H₁₁NO, 100%); MS (ESI) *m/z*: 414.2 (M + H)⁺, 827.8 (2M)⁺, 849.7 (2M + Na)⁺. Anal. Calcd for C₂₈H₄₇NO: C, 81.29; H, 11.45; N, 3.39. Found: C, 81.03; H, 11.33; N, 3.30.

References

- (1) Barton, D. H. R.; Day, M. J.; Hesse, R. H.; Pechet, M. M. A new rearrangement of ketonic nitrones; a convenient alternative to the Beckmann rearrangement. *J. Chem. Soc., Perkin Trans. 1* **1975**, 1764-1767.

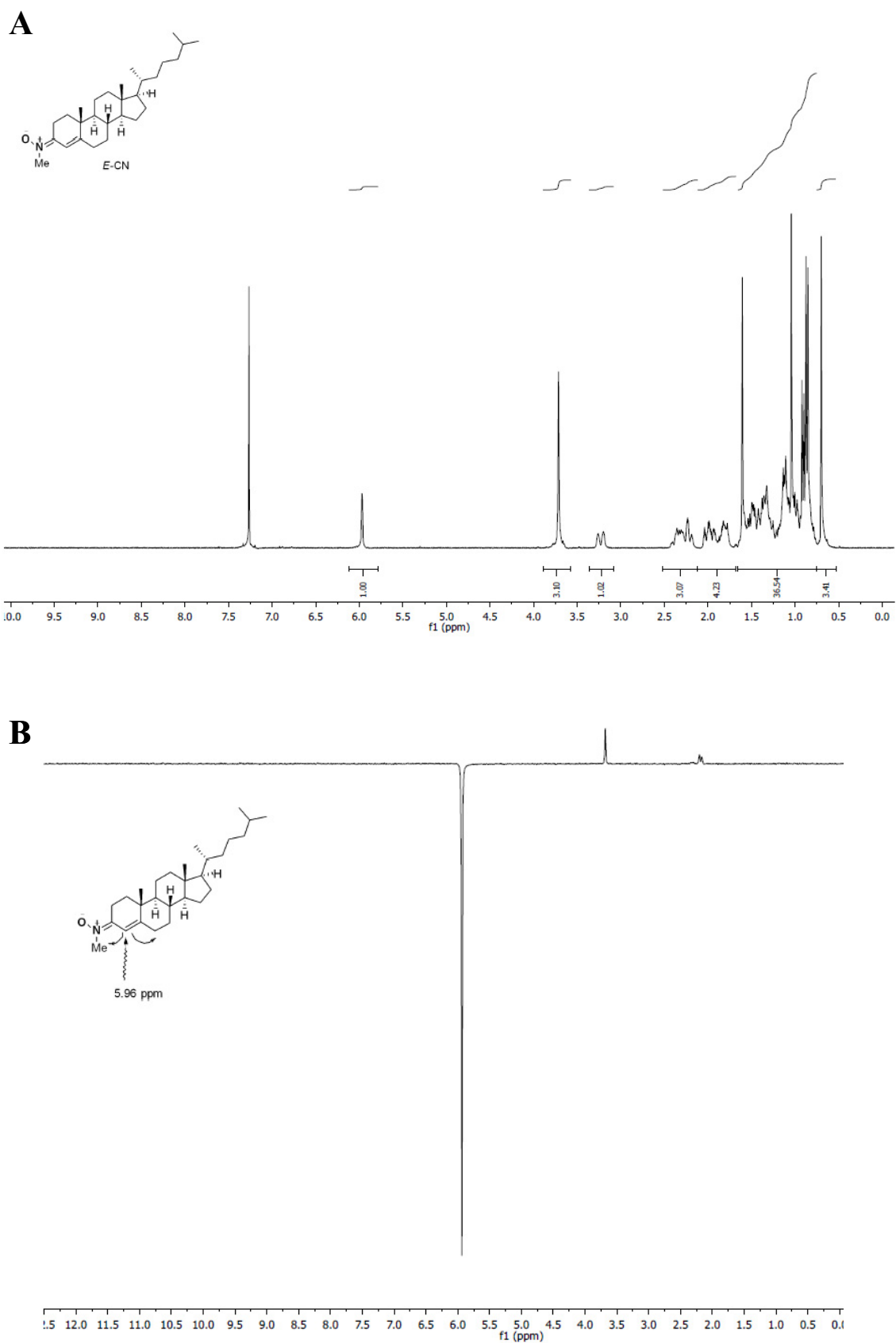


Figure 2. (A) ^1H NMR spectrum of ChN ISQ-201 . (B) Selective ^1H NMR nOe spectrum of ChN ISQ-201 .

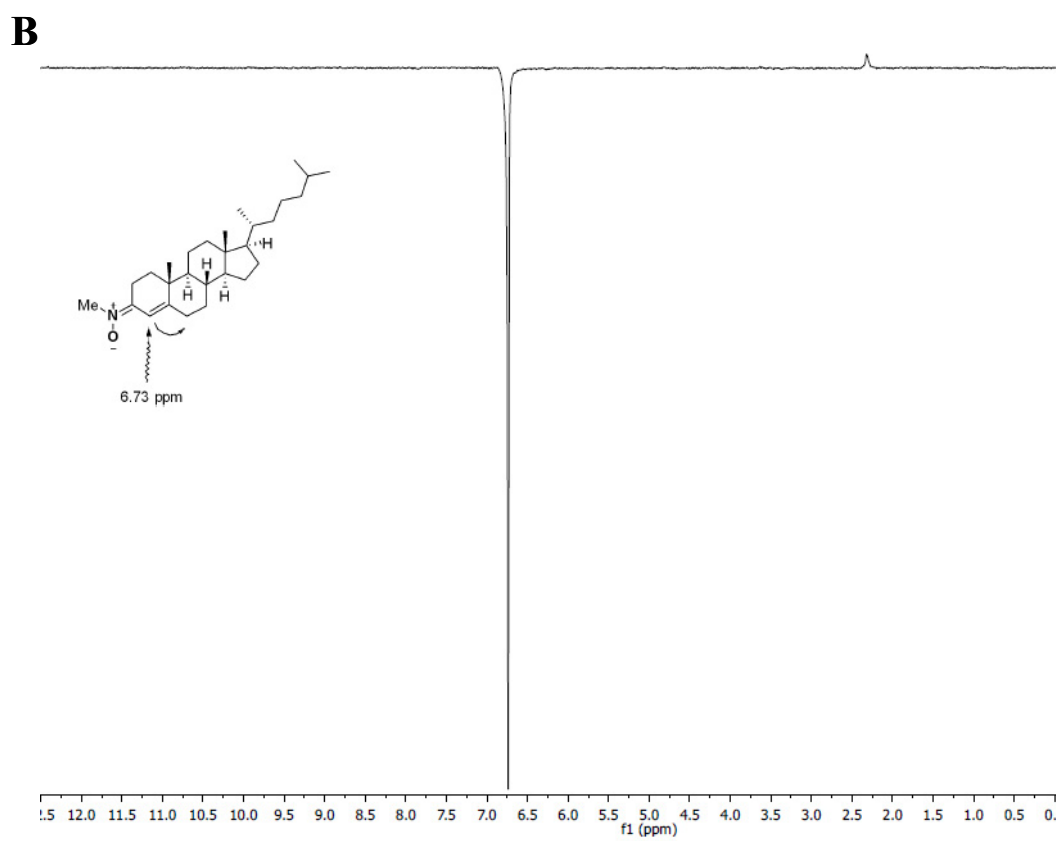
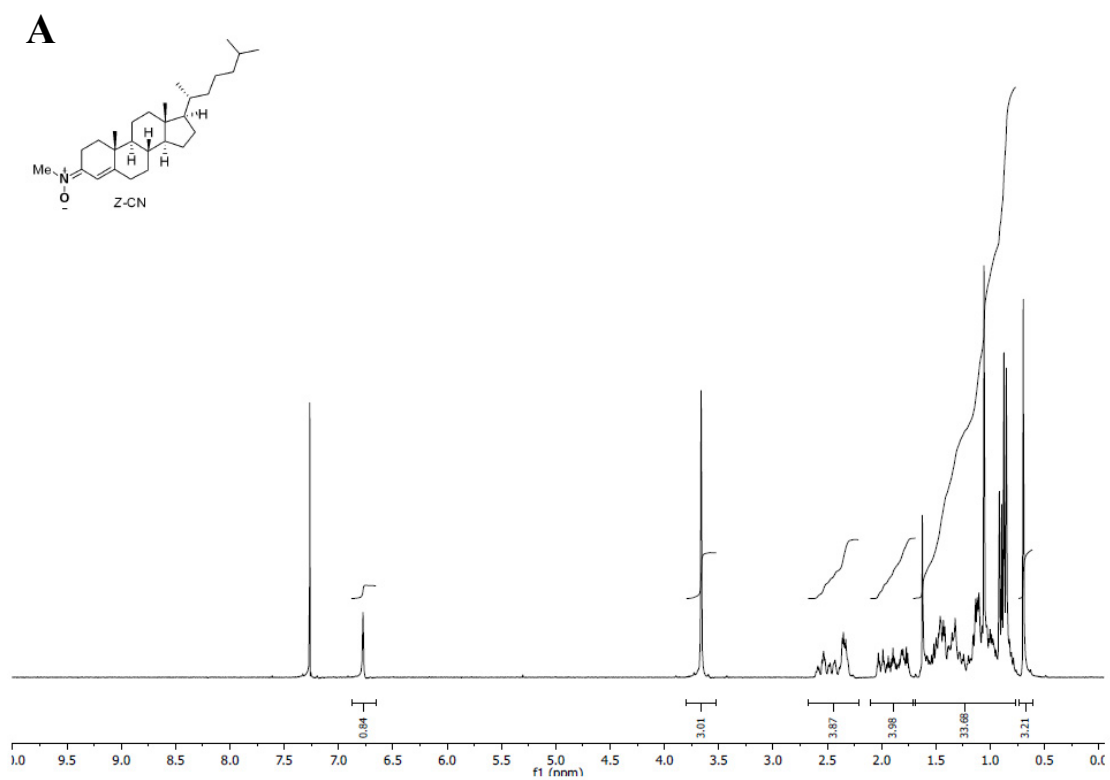
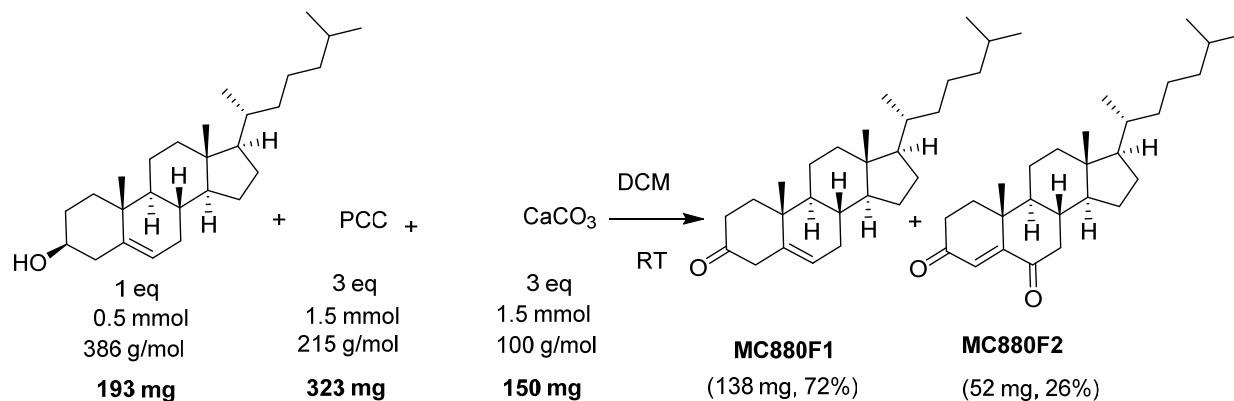


Figure 3. (A) ^1H NMR spectrum of ChN ISQ-202. (B) Selective ^1H NMR nOe spectrum of ChN ISQ-202.

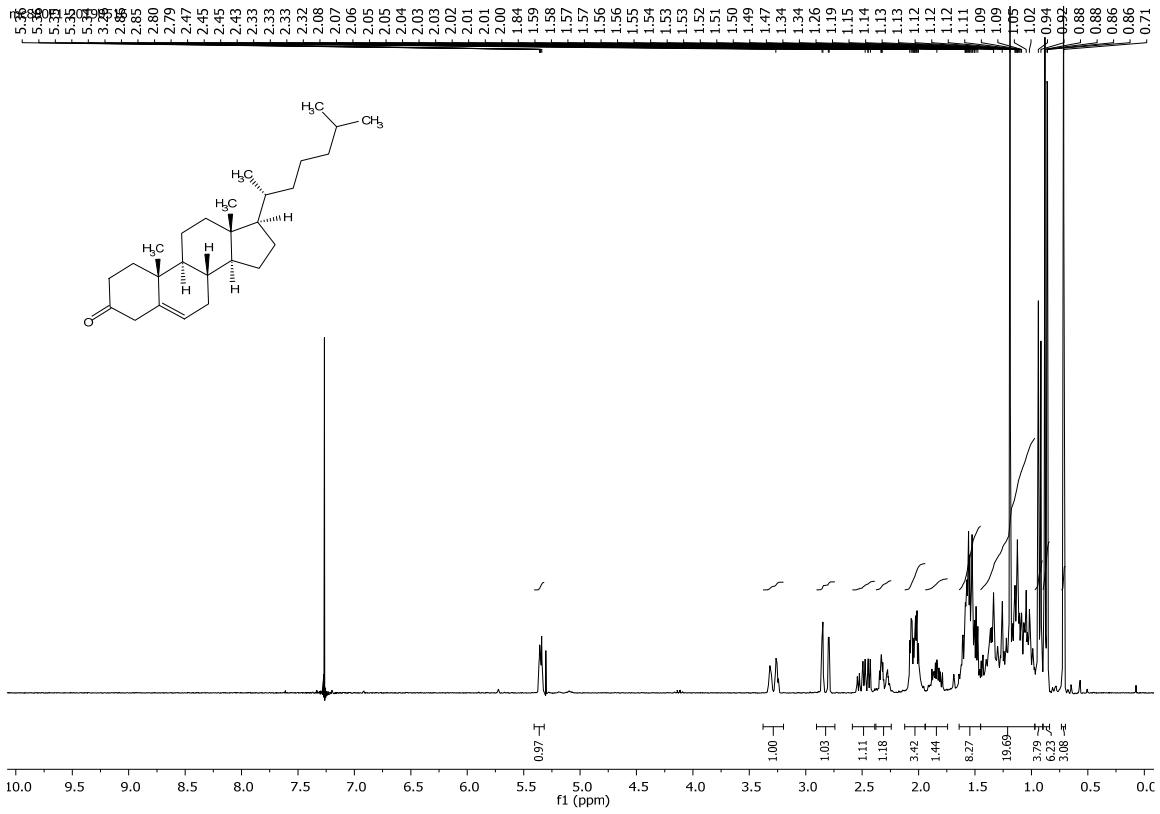
B. Synthesis of cholesteronitron ISQ-201 from natural cholesterol-plant

1. Oxidation of cholesterol

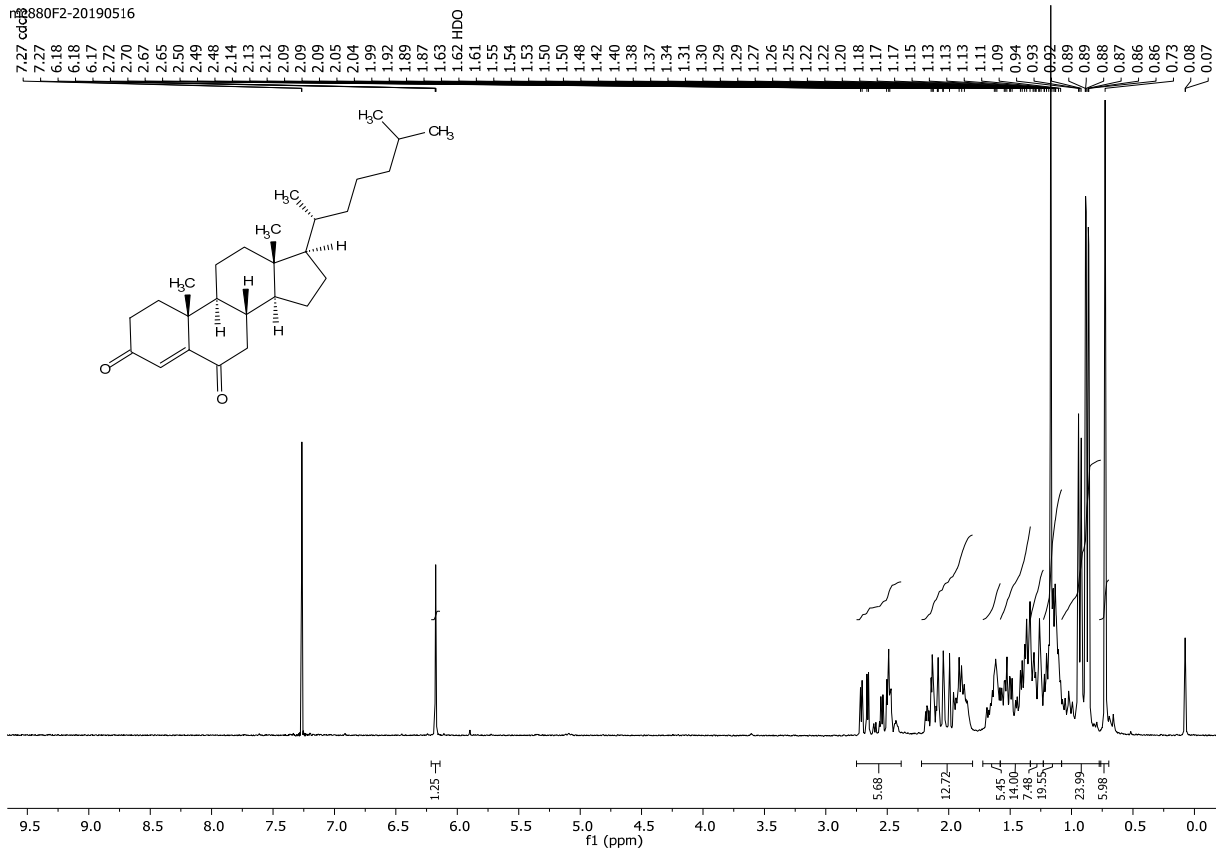


Following the described method (A. K. Batta, G. Saien, S. Shefer *Steroids* **1988**, *52*, 109-122), to a suspension of PCC (323 mg, 1.5 mmol, 3 equiv), CaCO₃ (150 mg, 1.5 mmol, 3 equiv) in DCM (10 mL), a solution of cholesterol-plant (193 mg, 0.5 mmol) in DCM (5 mL) was added. Then, the mixture was stirred at rt for 45 min, was filtered over Celite, washed with DCM, the solvent was evaporated, and the residue was recrystallized from methanol, affording a solid that was purified by column chromatography, using hexane/AcOEt (from 1% to 10%), to give: cholest-5-en-3-one (MC880F1, 138 mg, 72%), and cholest-4-en-3,6-dione (MC880ISQ-201, 52 mg (26%) (J.-G. Cui, L. Fan, L.-L. Huang, H.-L. Liu, A.-M. Zhou, *Steroids* **2009**, *74*, 62-72).

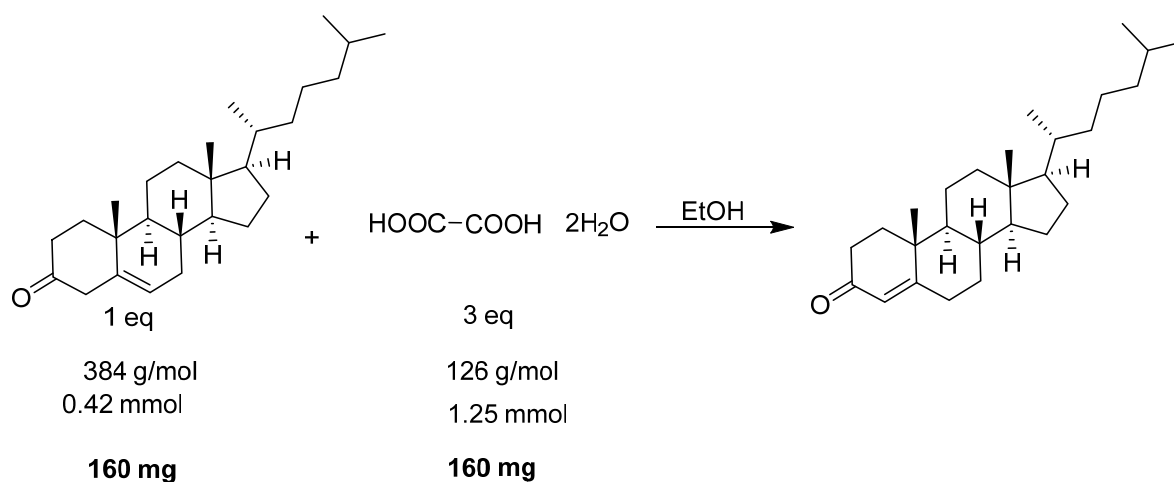
¹H NMR MC880F1



¹H NMR- MC880ISQ-201

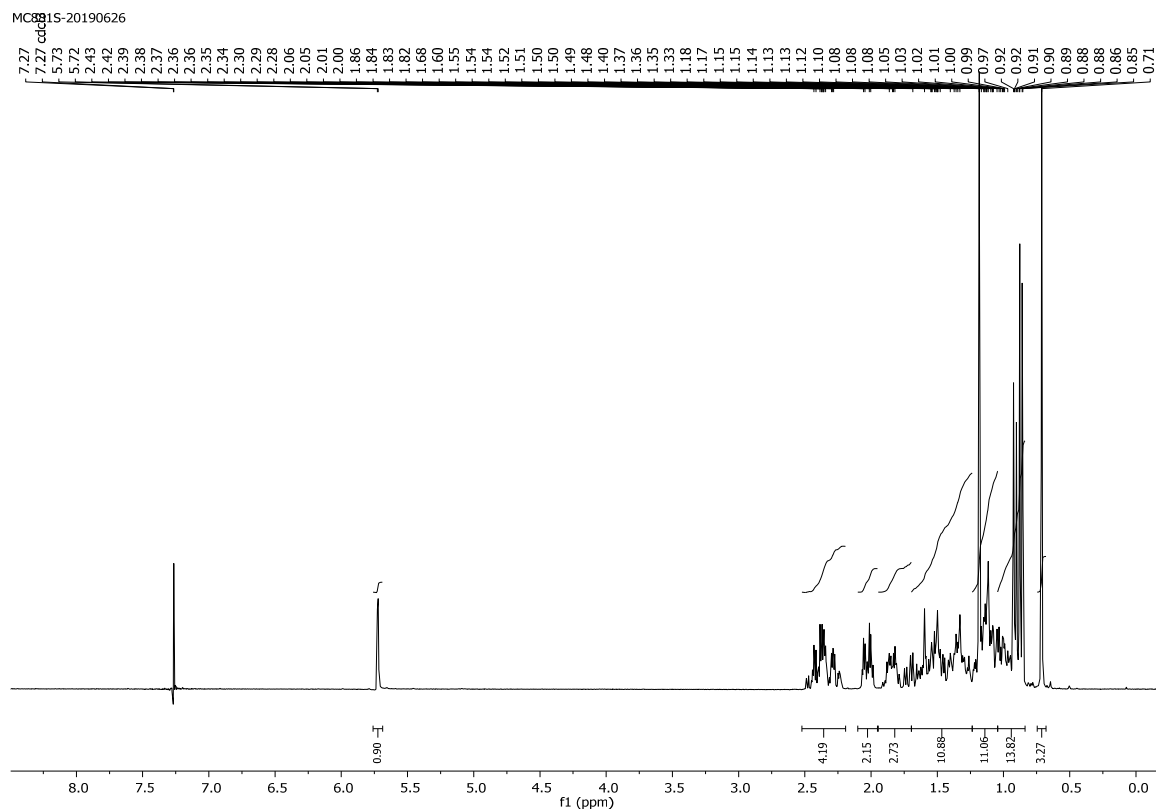


2. Isomerization of compound MC880F1

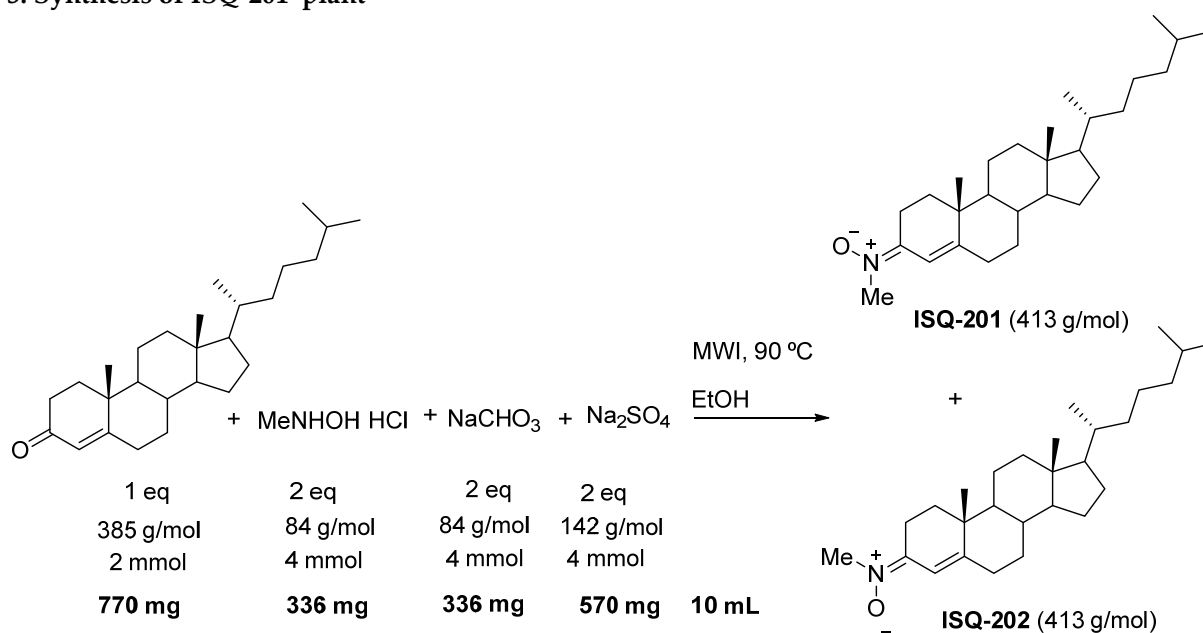


Following the described method (J.-G. Cui, L. Fan, L.-L. Huang, H.-L. Liu, A.-M. Zhou *Steroids* **2009**, *74*, 62-72), a solution of **MC880F1** (160 mg, 0.42 mmol) in EtOH (5 mL) was treated with oxalic acid monohydrate (160 mg, 1.25 mmol, 3 equiv), at reflux for 24 h. The mixture was cooled, the solvent evaporated, and the residue was dissolved in DCM, washed with water, and extracted several (x 3) times. The organic phase was dried with Na₂SO₄, filtered, and the solvent was evaporated to give pure **MC881F1** (155 mg, 97%).

¹H NMR- MC881F1



3. Synthesis of ISQ-201 plant



A suspension of cholest-4-en-3-one (**MC881F1**) (770 mg, 2 mmol), *N*-methyl hydroxylamine. HCl (336 mg, 4 mmol, 2 equiv), NaHCO₃ (336 mg, 2 mmol), and Na₂SO₄ (570 mg, 4 mmol) in ethanol (10 mL), was irradiated (250 W) for 3 h at 90 °C. After cooling the mixture, the solvent was removed, and the residue was purified by column chromatography, using DCM/methanol (from 1% to 3%) to give **ISQ-201** (410 mg, 49%), **ISQ-201 + 2ISQ-202** (25 mg, 3%) and **ISQ-202** (205 mg, 25%).

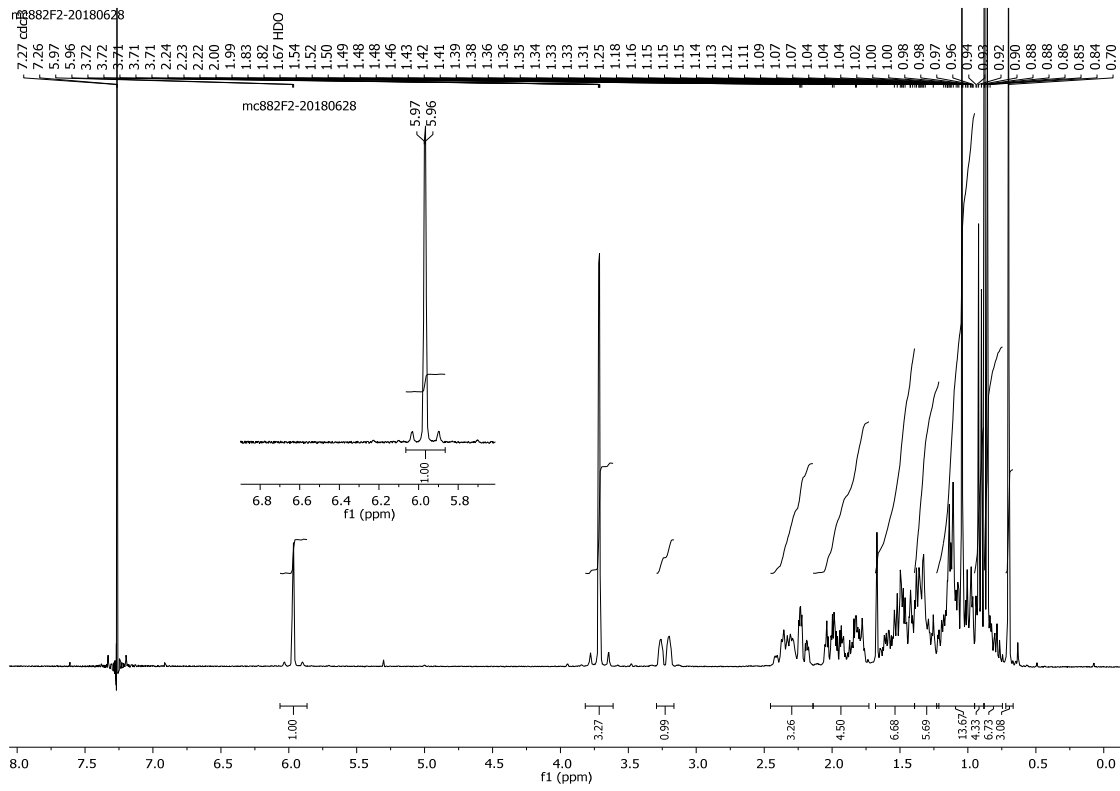
ISQ-201 : White solid; *R_f* (0.21, CH₂Cl₂/MeOH, 5%); mp 139-141 °C [*anti*(= *E*) isomer: mp 127-131 °C]; IR (KBr) ν max 2939, 2868, 2849, 1466, 1215 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 5.97 (d, *J* = 2.0 Hz, 1H, ⁴CH), 3.72 (s, 3H, NCH₃), 3.23 (d, *J* = 18.4 Hz, 1H, ²CH), 2.34 (m, 2H, ⁶CH₂), 2.21 (m, 1H, ²CH), 1.99 (m, 2H, CH₂), 1.80 (m, 2H, CH₂), 1.60 (s, 3H, CH₃), 1.36 (m, 10H, 5CH₂), 1.12 (m, 6H, 6CH₂), 1.04 (s, 3H, ¹⁹CH₃), 0.99 (m, 2H, CH₂), 0.91 (d, *J* = 6.4 Hz, 3H, ²¹CH₃), 0.88 (d, *J* = 1.3 Hz, 3H, ²⁶CH₃), 0.86 (m, 3H, ²⁷CH₃), 0.70 (s, 3H, ¹⁸CH₃); ¹³C NMR (101 MHz, CDCl₃) δ 156.8 (³C), 146.4 (⁵C), 112.9 (⁴CH), 56.1 (¹⁷CH), 55.9 (¹⁴CH), 53.5 (⁹CH), 46.0 (¹³C), 42.3 (NCH₃), 39.6 (C), 39.4 (C), 37.9 (¹⁰C), 36.1 (C), 35.77 (C), 35.73 (C), 34.4 (C), 33.4 (C), 32.2 (²⁵CH₂), 28.1 (¹⁶CH₂), 27.9 (²CH₂), 24.2 (¹⁵CH₂), 23.8 (²⁴CH₂), 22.7 (²⁶CH₃), 22.5 (²⁷CH₃), 21.4 (CH₂), 21.3 (¹¹CH), 18.6 (¹⁹CH₃), 17.8 (²¹CH₃), 11.9 (¹⁸CH₃). MS (EI) *m/z*: 413 (M, 37%)+, 398 (M-CH₃, 27%), 397(M-O,

70), 137(C₈H₁₁NO, 100%); MS (ESI) *m/z*: 414.2 (M + H)⁺, 436.2 (M + Na)⁺, 827.8 (2M)⁺, 849.7 (2M + Na)⁺.

Anal. Calcd for C₂₈H₄₇NO: C, 81.29; H, 11.45; N, 3.39. Found: C, 80.98; H, 11.29; N, 3.44.

ISQ-202: White solid; *R_f* (0.20, CH₂Cl₂/MeOH, 5%); mp 153-155 °C [*syn*(= *E*) isomer: mp 146-149 °C¹]; IR (KBr) *v*_{max} 2936, 2868, 1629, 1214 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 6.78 (s, 1H, ⁴CH), 3.66 (s, 3H, NCH₃), 2.44 (m, 4H, 2CH₂), 1.88 (m, 4H, 2CH₂), 1.37 (m, 14H, 7CH₂), 1.04 (s, 3H, ¹⁹CH₃), 0.98 (m, 2H, CH₂), 0.91 (d, *J* = 6.4 Hz, 3H, ²¹CH₃), 0.88 (d, *J* = 1.4 Hz, 3H, ²⁶CH₃), 0.85 (d, *J* = 1.4 Hz, 3H, ²⁷CH₃), 0.70 (s, 3H, ¹⁸CH₃); ¹³C NMR (101 MHz, CDCl₃) δ 123.7 (³C), 120.3 (⁵C), 113.7 (⁴CH), 56.0 (¹⁷CH), 55.9 (¹⁴CH), 53.5 (⁹CH), 46.4 (¹³C), 42.3 (NCH₃), 39.6 (C), 39.4 (C), 37.9 (¹⁰C), 36.0 (C), 35.72 (C), 35.71 (C), 35.4 (C), 32.9 (C), 32.2 (C), 28.1 (C), 27.9 (C), 24.1 (¹⁶CH₂), 23.7 (¹⁵CH₂), 23.6 (²⁴CH₂), 22.7 (²⁶CH₃), 22.5 (²⁷CH₃), 21.3 (¹¹CH₂), 18.6 (¹⁹CH₃), 17.8 (²¹CH₃), 11.9 (¹⁸CH₃). MS (EI) *m/z*: 413 (M, 37%)⁺, 398 (M-CH₃, 27%), 397 (M-O, 70), 137 (C₈H₁₁NO, 100%); MS (ESI) *m/z*: 414.2 (M + H)⁺, 827.8 (2M)⁺, 849.7 (2M + Na)⁺. Anal. Calcd for C₂₈H₄₇NO: C, 81.29; H, 11.45; N, 3.39. Found: C, 81.03; H, 11.33; N, 3.30.

¹H NMR ISQ-201



¹H NMR ISQ-202

