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Scutione, a New Bioactive Norquinonemethide Triterpene from *Maytenus scutioides* (Celastraceae)†

Antonio G. González,[a,*] Nelson L. Alvarenga,[a] Angel G. Ravelo,[a] Isabel L. Bazzocchi,[a] Esteban A. Ferro,[b] Angel G. Navarro[c] and Laila M. Moujir[c]

[a]*Instituto Universitario de Bio-Orgánica Antonio González, Universidad de La Laguna, Avda. Astrofísico F° Sánchez, 2, 38206, La Laguna, Tenerife, Canary Islands, Spain*

[b]*Departamento de Investigación, Universidad Nacional de Asunción, San Lorenzo, P.O. Box 1055, Paraguay*

[c]*Departamento de Microbiología y Biología Celular, Universidad de La Laguna, Tenerife, Canary Islands, Spain*

Abstract—Scutione (**1**), a new norquinonemethide triterpene with a netzahualcoyene type skeleton, has been isolated from the root bark of *Maytenus scutioides* (Celastraceae) by bioactivity-directed fractionation. The structure of **1** has been elucidated by means of ^{1}H and ^{13}C NMR spectroscopic studies, including ^{1}H–^{13}C heteronuclear correlation (HETCOR), a long-range correlation spectrum with inverse detection (HMBC) and ROESY experiments. Compound **1** showed antibiotic activity against Gram-positive bacteria and modest cytotoxic activity against HeLa, Hep-2 and Vero cell lines. Fluoride derivatives **2**–**4** were prepared and assayed for bioactivity, where **2** showed slight improvement of the cytotoxic potency. Copyright © 1996 Elsevier Science Ltd

Introduction

There are four different structures of norquinonemethide triterpenoids in the Celastraceae; those with non-extended conjugation as in 7α-hydroxy-7,8-dihydro-iguesterin;[1] those with an extended conjugation of the quinone methide A ring with a carbonyl group as in the case of dispermoquinone;[2] those with an additional double bond as in pristimerin[3] or tingenone;[4] and the quinones with an additional dienic system as with netzahualcoyone.[5]

All of these types of quinones have shown interesting biological properties and their antibiotic and cytostatic activities have been intensively studied; in addition, several studies on the structure–activity relationships and mechanisms of action of this type of triterpenes have been published.[6–9]

M. scutioides (Lourteig and O'Donnell[10]) is a subtropical shrub that is distributed in the central region of South America. In our research for new bioactive compounds, we investigated the root bark of *M. scutioides* for cytostatic and antibiotic constituents. The cytotoxicity and antibiotic tests were used to direct the fractionation. This paper reports the isolation and identification of five known triterpenes: β-amyrin, netzahualcoyene, tingenone, pristimerin, celastrol and a new norquinonemethide triterpene, scutione (**1**).

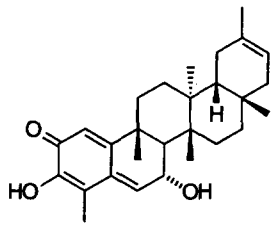
Given the potential effects of the introduction of fluorine into such organic compounds,[11] the bioactivity and structural elucidation of **1** and three fluoride derivatives (**2**–**4**) are reported.

Results and Discussion

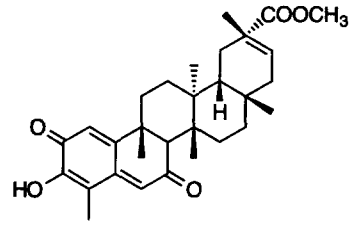
Scutione (**1**) was isolated as an amorphous reddish-brown powder with the molecular formula $C_{28}H_{34}O_3$ (HREIMS). Its IR spectrum indicated the presence of hydroxyl (3375 cm^{-1}) and carbonyl groups (1700 and 1590 cm^{-1}). The ^{1}H NMR spectrum (Table 1) showed an ABC system of three vinyl protons at δ 6.60 as a singlet and two doublets at δ 7.18 and δ 6.26, corresponding to H-1, H-6, and H-7 protons, characteristic of a triterpenic quinonoid system; signals for six methyls were also observed, one of them on an aromatic ring at δ 2.28 (Me-23), one on a double bond at δ 1.71 (Me-26) and another as a doublet at δ 1.10 (Me-30); also a multiplet (δ 2.47) was observed coupled with the signal at δ 1.10 (Me-30) and was assigned to H-20. All these data indicated that **1** was a norquinonemethide triterpene related to tingenone, but with a rearranged methyl and an additional conjugated double bond as in the netzahualcoyene skeleton. This was corroborated by the UV spectrum, with bands at 442, 262 and 238 nm.[5] The ^{13}C NMR spectrum (Table 2) and the HMQC and HMBC experiments (Table 3) confirmed the structure proposed. The stereochemistry of the methyl on C-20 (Me-30) was resolved by a ROESY experiment showing an NOE effect between the Me-27 and H-20 (Scheme 1).

Fluoride derivatives were prepared using *N*-fluoropyridinium triflate,[12] which is a useful fluorinating agent for highly active methylene compounds. When **1** was

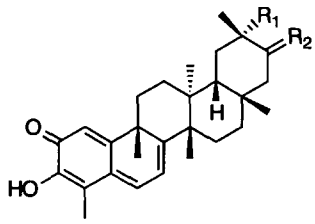
†This paper is dedicated to the late Professor W. S. Johnson, Stanford University (1908–1995).



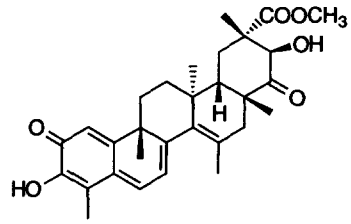
7α-Hydroxy-7,8-dihydro-iguesterin



Dispermoquinone



Pristimerin $R_1 = COOCH_3$, $R_2 = 2H$
Tingenone $R_1 = H$, $R_2 = O$

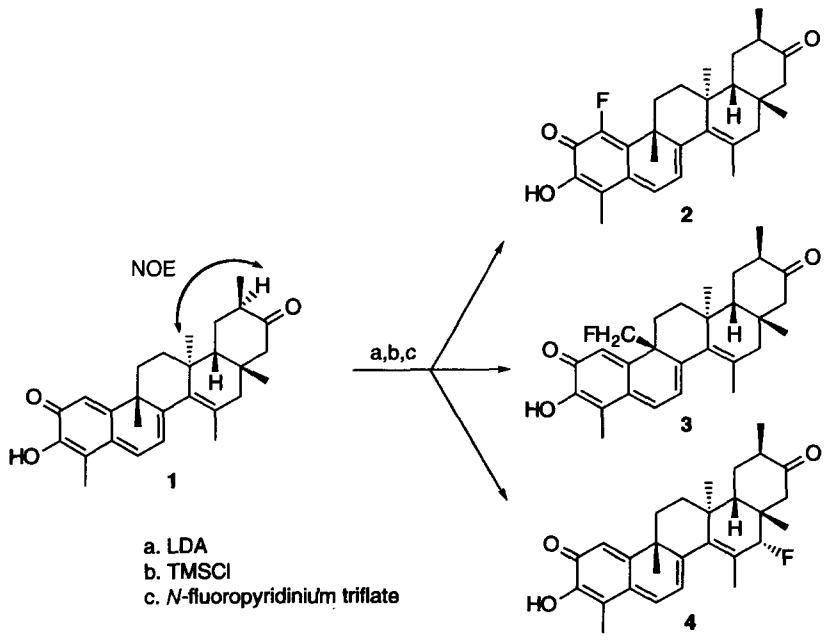


Netzahualcoyone

treated with this reagent, the derivatives, **2** (10% yield), **3** (7.5% yield) and **4** (8% yield), were obtained, but not the expected 21-fluorine derivative.

The derivative **2** was a red amorphous solid; its IR spectrum showed absorptions for hydroxyl (3400 cm^{-1}), carbonyl (1700 and 1590 cm^{-1}), and fluorine (1040 cm^{-1}) groups. The mass spectrum showed a molecular ion at *m/z* 437 ($M^+ + 1$) and a fragment at *m/z* 417 (M^+-19), suggesting the presence of a fluorine. Its ^1H NMR (Table 1) showed, as the most notable differences with respect to scutione (**1**) the disappearance of the signal at δ 6.60 (H-1) and the shift of the H-6 signal from δ 7.18 to δ 7.27. The ^{13}C NMR spectrum (Table 2) showed only two doublet aromatic carbons and a quaternary carbon at δ 138.5 as a doublet due to the ^{19}F–^{13}C coupling, and these signals were assigned to C-6, C-7 and C-1, respectively, by means of the HMBC and HMQC correlations (Table 3). All these data suggested that **2** was the derivative of **1** with a fluorine at C-1. The formation of the 1-fluorine-scutione could be explained by an aromatic electrophilic substitution mechanism.

Derivative **3** showed bands for hydroxyl, carbonyl and fluorine groups in its IR spectrum; the EIMS give



Scheme 1.

Table 1. ^{1}H NMR (200 MHz) data (δ, $CDCl_3$) of **1–4**

	1	2	3	4
H-1	6.60 s		6.65 s	6.60s
H-6	7.18 d (6.8)	7.27 d (6.9)	7.14 d (6.8)	7.18 d (6.7)
H-7	6.26 d (6.8)	6.21 d (6.9)	6.42 d (6.9)	6.28 d (6.8)
H-16				3.50 br s
H-20	2.47 m	2.48 m	2.45 m	
H-22	2.71 d (14.6)	2.73 d (14.5)	2.71 d (14.5)	
Me-23	2.28 s	2.30 s	2.27 s	2.28 s
Me-25	1.26 s	1.63 s		1.29 s
Me-26	1.71 s	1.69 s	1.84 s	1.89 s
Me-27	1.08 s	1.11 s	1.14 s	1.07 s
Me-28	0.98 s	0.99 s	0.98 s	1.04 s
Me-30	1.10 d (6.6)	1.11 d (6.5)	1.10 d (6.4)	1.12 d (6.4)

J values are given in Hz in parentheses.

fragments at *m/z* 416 (M^+-20, HF) and *m/z* 403 (M^+-33), suggesting the presence of a fluorinemethylene group. Its ^{1}H NMR spectrum (Table 1) showed, as differences with respect to **1**, the disappearance of the signal at δ 1.26 (Me-25) and a shift of the signal corresponding to Me-26 from δ 1.71 to δ 1.84; also notable was the presence of two doublets at δ 3.56 and δ 3.80, respectively. The ^{13}C NMR spectrum (Table 2) showed a signal for a methylene carbon at δ 53.2 as a triplet split due to the ^{19}F–^{13}C coupling, and an HMBC experiment confirmed that the fluorinemethylene group was on C-9. The formation of the 9-fluorinemethylene-scutione could be explained by the known association between pyridinium salts and quinones,[13] by an unknown mechanism.

Table 2. ^{13}C NMR (50 MHz) data (δ, $CDCl_3$) of **1–4**

	1	2	3	4
C-1	120.0 d	138.5 s	121.7	120.2
C-2	178.1 s	172.7 s	178.5 s	178.4 s
C-3	146.3 s	145.9 s	146.8 s	146.4 s
C-4	116.8 s	117.7 s	117.6 s	116.7 s
C-5	128.1 s	127.5 s	130.0 s	128.5 s
C-6	134.4 d	137.0 d	134.2 d	133.7 d
C-7	122.1 d	121.2 d	126.2 d	121.6 d
C-8	158.6 s	152.1 s	152.7 s	158.2 s
C-9	44.2 s	47.0 s	48.6 s	44.6 s
C-10	159.7 s	160.1 s	155.0 s	160.5 s
C-11	36.0 t	28.5 t	34.5 t	36.2 t
C-12	31.8 t	31.8 t	32.1 t	38.4 t
C-13	42.1 s	41.7 s	42.4 s	40.6 s
C-14	135.6 s	136.0 s	134.7 s	139.8 s
C-15	127.9 t	128.0 t	130.4 t	129.3 t
C-16	45.6 t	45.4 t	46.1 t	76.4 d
C-17	36.3 s	36.4 s	36.5 s	29.7 s
C-18	47.8 d	47.6 d	48.1 d	42.8 d
C-19	37.8 t	37.3 t	37.9 t	31.5 t
C-20	41.7 d	41.7 d	42.0 d	42.2 d
C-21	213.8 s	213.8 s	213.9 s	212.8 s
C-22	49.8 t	50.1 t	50.3 t	47.9 t
C-23	10.4 c	11.0 c	10.7 c	10.4 c
C-25	28.5 c	21.1 c	53.2 t	28.3 c
C-26	21.3 c	18.5 c	21.9 c	19.5 c
C-27	23.1 c	23.0 c	30.7 c	22.7 c
C-28	30.2 c	30.4 c	23.6 c	24.6 c
C-30	16.0 c	16.0 c	16.3 c	16.3 c

Values based on ^{13}C–^{1}H, HMBC and DEPT experiments.

Derivative **4** was obtained as a red amorphous solid; its IR spectrum showed bands for hydroxyl (3400 cm^{-1}), carbonyl (1700 and 1590 cm^{-1}) and fluorine (1080 cm^{-1}) groups and the EIMS showed a fragment at *m/z* 416 (M^+-20). Its ^{1}H NMR spectrum (Table 1) showed a broad singlet at δ 3.50 assignable to the halogen and the Me-26 was shifted, with respect to **1**, from δ 1.71 to 1.89. The HMBC experiment (Table 3) showed two-bond coupling between a geminal proton to the halogen and C-15 and three-bond couplings with C-14 and C-18; also three-bond couplings were observed between the protons on C-26 and the carbon bearing the halogen (δ 76.38) and with the protons on C-28. These data suggested that **4** was a derivative of **1** with a fluorine on C-16; the stereochemistry was established as α by a ROESY experiment (Scheme 1) showing an NOE effect between the methyl-28 and the proton geminal to the halogen, together with a Dreiding model and mechanical molecular calculations. The formation of this derivative could be explained by the abstraction of a proton on C-16, enolization of the carbonyl group on C-2 with rearrangement of the double bonds and recovery of the methylene–quinone system followed by electrophilic addition of the fluorine to the double bond at C_{15}–C_{16}.

Antibiotic assays

Antimicrobial effects were obtained using a disk diffusion test.[6] The minimal inhibitory concentrations were determined only for those compounds that had previously shown an inhibition zone in the disk diffusion test. MIC values listed in Table 4 clearly show that the effect of scutione (**1**) and its fluoride derivatives **2–4** is limited to Gram-positive bacteria and they are inactive against Gram-negative bacteria and the yeast, *Candida albicans*, at <20 μg/mL.

We have reported in a previous paper the antimicrobial activities of some scutione-related triterpenoquinones isolated from different species belonging to the Celastraceae.[8] These products differ in the functional groups in ring E, for example, netzahualcoyone differs from scutione in the presence of a methyl ester group on C-20, a hydroxyl group on C-21 and a carbonyl group on C-22. Scutione was more active indicating that the position of the ketonic group is relevant for the activity or the presence of the other groups results in a decrease in the antibacterial potency.

Another aspect to be considered is the presence of a double bond at C_{14}–C_{15}. To obtain information about its role in the activity, we compared the MIC of scutione (**1**) with that of tingenone, whose ring E is identical to that in scutione, but it lacks of the double bond in ring D. The MIC of tingenone against *Bacillus subtilis* is three-times higher than that of scutione.[14]

This observation indicates that the double bond is also important for the activity.

In spite of the expectation that the presence of fluorine might improve the biological properties of some compounds, data in Table 4 show that the three fluoride derivatives have less antibiotic activity than scutione (**1**).

Table 3. Three bond $^{1}H-^{13}C$ couplings in compounds **1–4**

	Irradiated protons	Observed carbons
1	H-1	C-3, C-5, C-9
	H-6	C-4, C-8, C-10
	H-7	C-5, C-9, C-14
	CH_3 (23)	C-3, C-4,[a] C-5
	CH_3 (26)	C-14, C-15,[a] C-16
	CH_3 (30)	C-19, C-20,[a] C-21
2	H-6	C-4, C-8, C-10
	H-7	C-5, C-9, C-14
	CH_3 (23)	C-3, C-4,[a] C-5
	CH_3 (26)	C-14, C-15,[a] C-16
	CH_3 (30)	C-19, C-20,[a] C-21
3	H-1	C-3, C-5, C-9
	H-6	C-4, C-8, C-10
	H-7	C-5, C-9, C-14
	2H-25	C-8, C-10
	CH_3 (23)	C-3, C-4,[a] C-5
	CH_3 (26)	C-14, C-15,[a] C-16
	CH_3 (30)	C-19, C-20,[a] C-21
4	H-1	C-3, C-5, C-9
	H-6	C-4, C-8, C-10
	H-7	C-5, C-9, C-14
	H-16	C-14, C-15,[a], C-18
	CH_3 (23)	C-3, C-4,[a] C-5
	CH_3 (26)	C-14, C-15,[a] C-16
	CH_3 (28)	C-16, C-18, C-22
	CH_3 (30)	C-19, C-20,[a] C-21

[a]Two-bond coupling enhancement observed.

Table 4. Minimal inhibitory concentrations (μg/mL) of **1–4** against Gram-positive bacteria

Bacteria	1	2	3	4	Control
S. aureus	2	10–15	15	3–4	2–5
S. albus	0.5	2.8	3.6	2.7	1
S. epidermidis	0.2–0.3	0.5	1.5–2	2.7	5
S. warnieri	2	n.a.	n.a.	n.a.	n.a.
S. saprophyticus	1–2	n.a.	n.a.	n.a.	4
M. luteus	0.6	15	2	2.7	1
B. subtilis	0.1–0.16	0.2	0.7–0.9	1–1.5	2–5
B. pumilus	0.2	0.4–0.6	1.5–2	2.5–3	>10
B. alvei	0.3	n.a.	n.a.	n.a.	8
B. megaterium	0.2–0.4	n.a.	n.a.	n.a.	n.a.
B. cereus	0.1–0.2	n.a.	n.a.	n.a.	n.a.

n.a.: not assayed; all assays were carried out in triplicate; all compounds were inactive against the eight Gram-negative bacteria assayed (MIC>20 μg/mL); cephotaxime was used as the positive control.

Table 5. Cytotoxic activity of **1–4** against HeLa, Hep-2 and Vero cells

	IC_{50} (μg/mL)				
Cell line	1	2	3	4	Control
HeLa	4.9	2.6	24	>40	0.1
Hep-2	5.6	9	>40	>40	0.6
Vero	7.2	5.2	34	>40	n.a.

Mercaptopurine was used as the positive control; n.a.: not assayed; results are mean values from three determinations.

Table 5 shows the cytotoxic activity of compounds **1–4** against three cell lines in culture. As a rule, activity against HeLa cells was stronger than the other cell lines. None of these comporids (**1–4**) showed significant activity as compared with that of mercaptopurine (0.1 μg/mL), which was used as a positive control in this study. Introduction of fluorine resulted again in a decrease of the activity, except for **2** against the HeLa and Vero cell lines when a slight increase was observed.

Experimental

General

Melting points are uncorrected. IR spectra were taken on a PE 681 spectrophotometer. ^{1}H and ^{13}C NMR spectra were taken on a Bruker WP-200 SY in $CDCl_3$ (at 200 and 50 MHz, respectively), while the HMBC was taken on a Bruker at 400 MHz. *J* values are given in Hz. Optical rotations were measured on a Perkin–Elmer 241 automatic polarimeter and $[\alpha]_D$ values are given in 10^{-1} °/cm/g; EIMS were recorded on a VG Micromass LTD-ZAB-2F and/or on an HP 5930 A at 70 eV. UV spectra were run on a Perkin–Elmer 550 SE.

Plant material

The plant was gathered in Paraguay, in December 1993, and a voucher specimen (no. R. Degen 3117) is on file with the Herbarium of the Departamento de Botánica, Facultad de Ciencias Químicas, Universidad Nacional de Asunción, Paraguay.

Bioassays

The extracts, fractions and compounds isolated from the title plant were routinely evaluated for antibiotic and cytotoxic activities.

Antibiotic assay

The activity of the compounds were tested against Gram-positive (*Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* CECT 232, *Staphylococcus albus* SAB C1, *Staphylococcus warneri* CECT 236, *Staphylococcus saprophyticus* CECT 235, *Bacillus subtilis* CECT 39, *Bacillus pumilus* CECT 29, *Bacillus alvei* CECT 2, *Bacillus megaterium* CECT 44, *Bacillus*

cereus CECT 496, *Micrococcus luteus* CECT 241) and Gram-negative (*Escherichia coli* CECT 99, *Salmonella* sp. CECT 456, *Salmonella typhimurium* STBC 1, *Pseudomonas aeruginosa* AK 958, *Pseudomonas fluorescens* ATCC 13525, *Proteus mirabilis* CECT 170, *Klebsiella pneumoniae* CECT 367, *Enterobacter hafniae* CECT 400) bacteria and the yeast (*Candida albicans* UBC1). The bacteria were maintained on Nutrient Agar (Oxoid) and the yeast on Sabouraud Agar (Oxoid) at 37 °C.

The minimal inhibitory concentration (MIC) of products previously dissolved in DMSO (dimethyl sulfoxide) exhibiting activity in the antimicrobial assays in agar media was estimated in liquid medium following the method of Buttiaux et al.[15]

Cytotoxic activity

HeLa (human carcinoma of the cervix), Hep-2 (human carcinoma of larynx) and Vero (African green monkey kidney) cell lines were grown as a monolayer in Dulbecco's modified Eagle's medium, DMEM (Gibco), supplemented with 10% new-born calf serum (Gibco) and 1% of penicillin–streptomycin mixture (10.000 IU/mL). The cells were maintained at 37 °C in 5% CO_2 and 90% humidity. The cytotoxicity, in vitro, was assessed using the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] reduction assay.[16] The optical density of each well was measured using a micro ELISA reader at 600 nm. The percentage of cell viability was plotted against the fraction concentrations and 50% cell viability (IC_{50}) was calculated from the curve. Experiments were repeated at least three times.

Extraction and isolation

The root bark (3 kg) of the plant was extracted with *n*-hexane:Et_2O (1:1, 3 L) in a Soxhlet apparatus. The extract (115 g) was repeatedly chromatographed on Sephadex LH-20 and Si gel using as solvents mixtures of *n*-hexane:$CHCl_3$:MeOH (2:1:1) and of *n*-hexane:EtOAc, respectively, to afford β-amyrin (5 mg), netzahualcoyene (15 mg), tingenone (40 mg), pristimerin (55 mg), celastrol (20 mg) and scutione (**1**) (17 mg).

Scutione (1). Amorphous solid, $[\alpha]_D^{20}$ +598.10 (*c* 1.25); UV λ_{max} nm: 442, 262, 238; IR ν_{max} cm^{-1}: 3.375, 3.000, 1.700, 1.590, 1.540, 1.440, 1.370, 1.280, 1.215, 1.190, 1.070, 850, 655; ^1H NMR δ: see Table 1; ^{13}C NMR δ: see Table 2; EIMS *m/z* (%): 418 (M^+, 9), 391 (1), 346 (3), 331 (5), 294 (4), 261 (6), 227 (7), 165 (32), 157 (29), 95 (28), 91 (24), 55 (100); HREIMS: *m/z* 418.250824 (calcd for $C_{28}H_{34}O_3$, 418.250795).

Fluorination of scutione (1)

In a dry two-necked round-bottom flask, provided with an addition funnel, rubber septa and thermometer, was placed under nitrogen di-isopropylamine (0.14 mmol) in 20 mL of dry THF; this mixture was then cooled in an ice bath before the slow addition of 0.13 mmol of *n*-BuLi (ca. 1.6 M in *n*-hexane) and the mixture was stirred at 0 °C for 10 min. The resulting solution was then cooled to −78 °C for 20 min before the dropwise addition of 0.13 mmol of scutione (**1**) in 20 mL of THF. Then 0.13 mmol of chlorotrimethylsilane was added and the solution was allowed to warm slowly at room temperature, stirred for 45 min and quenched by the addition of a saturated sodium bicarbonate solution. The crude extract was extracted with ethyl acetate, the combined organic layers were dried and the solvent was removed under vacuum. The residue was dissolved in 25 mL CH_2Cl_2 and 0.2 mmol of *N*-fluoropyridinium triflate was added and refluxed for 24 h. The crude extract was chromatographed using preparative plates to afford the pure products **2** (3.6 mg, 10% yield), **3** (5 mg, 7.5% yield), and **4** (3.7 mg, 8% yield).

1-Fluorine-scutione (2). Amorphous solid (3.6 mg), mp 62–63 °C, $[\alpha]_D^{20}$ +250.28 (*c* 0.36); UV λ_{max} nm: 455, 275, 238; IR ν_{max} cm^{-1}: 3600, 3400, 2950, 2900, 1700, 1590, 1510, 1430, 1375, 1270, 1220, 1075, 1040, 875, 660; ^1H NMR δ: 7.04 (1H, br s, int. with D_2O), for other signals, see Table 1; ^{13}C NMR δ: see Table 2; EIMS *m/z* %: 437 (M^++1, 14), 417 (13), 331 (34), 261 (30), 201 (18), 149 (100; p.b.), 69 (57).

9-Fluorinemethylene-scutione (3). Amorphous solid (5 mg), mp 75–76 °C; $[\alpha]_D^{20}$ +822.40 (*c* 0.5); UV λ_{max} nm: 437, 265; IR ν_{max} cm^{-1}: 3625, 3400, 2950, 2900, 1700, 1590, 1505, 1395, 1375, 1280, 1220, 1040, 875; ^1H NMR δ: 3.56–3.80 (J=10.4, 10.5 Hz), 7.03 (1H, br s int. with D_2O), for other signals, see Table 1; ^{13}C NMR δ: see Table 2; EIMS *m/z* %: 416 (M^+-20, 11), 403 (5), 31 (5), 279 (8), 251 (6), 227 (8), 201 (13), 149 (100; p.b.), 69 (20).

16α-Fluorine-scutione (4). Amorphous solid (3.7 mg), mp 100–101 °C; $[\alpha]_D^{20}$ +175.41 (0.37 g/mL); UV λ_{max} nm: 456, 263, 240; IR ν_{max} cm^{-1}: 3400, 2900, 1700, 1590, 1435, 1370, 1275, 1080, 920, 800, 660; ^1H NMR δ: 7.09 (1H, br s, int. with D_2O), for other signals, see Table 1; ^{13}C NMR δ: see Table 2; EIMS *m/z* %: 416 (M^+-20, 87), 401 (28), 331 (41), 292 (15), 227 (23), 201 (25), 149 (100; p.b.), 69 (68).

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