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A SARS-Coronovirus 3CL Protease Inhibitor Isolated from the Marine Sponge *Axinella* cf. *corrugata*: Structure Elucidation and Synthesis

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Experimental

General experimental procedures

UV spectra were recorded on a Hitachi U-3210 spectrophotometer. IR spectra (film on Si plate) were recorded on a FT-IR Bomem MB102 infrared spectrometer. NMR spectra were recorded either on a Bruker ARX 9.4 T instrument, operating at 400.35 MHz for ^{1}H and 100.10 MHz for ^{13}C channels, respectively, or on a Bruker Avance 300 Spectrometer with Bruker QNP 5 mm probe 300.00 MHz for ^{1}H and 75.0 MHz for ^{13}C, respectively. All NMR spectra were obtained at 25 ^{o}C using TMS as internal reference. Low and high resolution ESI-QIT-MS were recorded on a Bruker-Hewlett Packard 1100 Esquire-LC system mass spectrometer. Solvents used for extraction and flash chromatography were glass distilled prior to use. HPLC-grade solvents were utilized without further purification in HPLC separations. TLC analyses were performed with plastic-backed Si gel TLC sheets, eluting with different mixtures of MeOH and CH_2Cl_2. Plates were visualised under UV and also using phosphomolybdic acid as spray reagent, followed by heating at 100 ^{o}C. HPLC separations were performed either with a Waters quaternary pump 600, double beam UV detector 2487, and data module 746, or with a Waters autosampler 717, Waters 600 pump, Waters 2996 photodiode array detector monitored by Waters Millenium 32.

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Animal Material

The sponge *Axinella* cf. *corrugata* sp. was collected in several sites in the São Sebastião Channel, São Paulo, Brazil during the summer of 1995 and immediately stored in EtOH at –20 ^{o}C until processed. A voucher specimen was deposited at the Museu Nacional of the Universidade Federal do Rio de Janeiro (MNRJ 1749).

*Isolation of compounds **1** and **2** from the sponge Axinella cf. corrugata*

The sponge (*ca*. 2.0 kg wet weight) was separated from the EtOH (3 L) in which the sponge samples were stored, triturated in MeOH (3 L) in a waring blender and left overnight. After filtration of the MeOH extract, the solid material was re-extracted with MeOH (3 L). Both EtOH (3 L) and MeOH (6 L) extracts were pooled and evaporated until 500 mL of an aqueous suspension was obtained. The H_2O phase was partitioned with EtOAc. The EtOAc extract (0.574 g) was subjected to a chromatography on Sephadex LH-20 (MeOH), to give eight fractions. Fraction EtOAc-4 was subjected to a chromatography on a Waters silica gel Sep Pak column (10 g), eluted with a gradient of 1:1 EtOAc-MeOH in CH_2Cl_2, to give six fractions (EtOAc-4A to -4F). Fractions EtOAc-4C and EtOAc-4E were further purified by HPLC, using a Waters µBondapak Phenyl column (125 Å, 7.8 × 300 mm), using 2:3 MeOH/H_2O (containing 0.1% TFA) as the eluent, to give 3.9 mg of esculetin-4-carboxylic acid methyl ester (**1**, 0.20 10^{-3}%) and 4.5 mg of esculetin-4-carboxylic acid ethyl ester (**2**, 0.22 10^{-3}%).

*Esculetin-4-carboxylic acid methyl ester (**1**)*

Green amorph solid. UV (MeOH) λ_{max}/ nm: 207, 239, 271 and 375; IR (film) ν_{max}/ cm^{-1}: 3400-3000 (broad), 1709, 1619, 1566, 1447, 1384, 1283, 1202, 1142; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 10.44 (1H, s, OH-6), 9.60 (1H, s, OH-7), 7.46 (1H, s, H-8), 6.80 (1H, s, H-5), 6.60 (1H, s, H-3), 3.91 (3H, s, CH_3); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 164.4 (C-11), 160.0 (C-2), 150.9 (C-6), 148.8 (C-7), 143.0 (C-9), 142.5 (C-10), 113.5 (C-3), 110.3 (C-8), 107.0 (C-4), 102.9 (C-5), 53.0 (C-12); Positive HRTOF/ESI-MS *m/z* 495.0544 $[2\times M+Na]^+$ (Calc. for $C_{22}H_{16}O_{12}Na$ 495.0539).

*Esculetin-4-carboxylic acid ethyl ester (**2**)*

Green amorph solid; UV (MeOH) λ_{max}/ nm: 207, 239, 271 and 375; IR (film on a Si plate) ν_{max}/ cm^{-1}: 3400-3000 (broad), 1709, 1619, 1566, 1447, 1384, 1283, 1202, 1142; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 10.43 (1H, s, OH-6), 9.62 (1H, s, OH-7), 7.46 (1H, s, H-8), 6.80 (1H, s, H-5), 6.58 (1H, s, H-3), 4.37 (2H, q, 7 Hz, CH_2-12), 1.33 (3H, t, 7Hz, CH_3-13); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 163.9 (C-11), 160.0 (C-2), 150.9 (C-6), 148.8 (C-7), 143.0 (C-9), 142.8 (C-10), 113.3 (C-3), 110.2 (C-8), 107.0 (C-4), 102.9 (C-5), 62.0 (C-12), 13.7 (C-13); HRESIMS *m/z* 523.0843 $[2\times M+Na]^+$ (Calc. for $C_{24}H_{20}O_{12}Na$ 523.0852).

*Synthesis of esculetin-4-carboxylic acid ethyl ester (**2**). Preparation of 3-hydroxy-4-methoxyphenyl formate (**6**)*

A mixture of isovanillin (**5**) (760 mg, 5.0 mmol) and *m*-CPBA (1.5 equiv.) and $NaHCO_3$ (1.5 equiv.) in CH_2Cl_2 (25 mL) was stirred at rt for 2 h. The solids were removed by filtration and washed with CH_2Cl_2. The filtrate was extracted with an aqueous solution of $NaHCO_3$ and brine. The organic phase was dried over $MgSO_4$, filtered and evaporated under reduced pressure. The crude was chromatographed on silica gel (20% EtOAc in hexanes) to provide formate **6** (583 mg) in 70% yield.

*3-Hydroxy-4-methoxyphenyl formate (**6**)*

^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.23 (1H, s), 6.79 (1H, d, *J* 8.7 Hz), 6.70 (1H, d, *J* 2.6 Hz), 6.59 (1H, dd, *J* 8.7, 2.6 Hz), 5.84 (1H, bs), 3.84 (3H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 159.7, 146.2, 144.8, 143.7, 111.9, 110.7, 108.0, 56.1; Positive HRESIMS $[M+H]^+$ *m/z* 169.0505 ($C_8H_9O_4$, Calc. 169.0501).

*Preparation of compound **7***

A mixture of **6** (1.32 g, 7.8 mmol), sodium diethyloxalacetate (SDO, 2.0 g, 9.36 mmol) and H_3PO_4 (85%, 5 mL) was heated at 100 °C for 2 h. The mixture was then poured into ice and the solids collected by filtration and washed with H_2O. This crude product was purified by silica gel column chromatography (5% MeOH in CH_2Cl_2) affording 947 mg of **7** (3.6 mmol, 46% yield). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.82 (1H, s), 6.91 (1H, s), 6.83 (1H, s), 6.18 (1H, s), 4.42 (2H, q, *J* 7.0 Hz), 3.95 (3H, s), 1.41 (3H, t, *J* 7.0 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 164.1, 160.8, 150.6, 149.9, 144.0, 141.6, 119.1, 108.5, 106.5, 103.0, 62.2, 56.2, 13.9; Positive HRESIMS $[M+Na]^+$ *m/z* 287.0527 ($C_{13}H_{12}O_6Na$ Calc. 287.0732).

*Preparation of esculetin-4-carboxylic acid ethyl ester (**2**)*

To a solution of **7** (25 mg, 0.095) in CH_2Cl_2 was added at –78 °C a solution of BBr_3 (3.0 equiv., 1 mol L^{-1} in CH_2Cl_2). The mixture was allowed to warm to rt and silica gel was then added. Concentration under reduced pressure and chromatography on silica gel (5% MeOH in CH_2Cl_2) afforded **2** (20 mg) in 84% yield. HRESIMS *m/z* 523.0850 $[2xM+Na]^+$ (Calc. for $C_{24}H_{20}O_{12}Na$ 523.0852), $[M+Na]^+$ *m/z* 273.0353 ($C_{12}H_{10}O_6Na$, Calc. 273.0375).

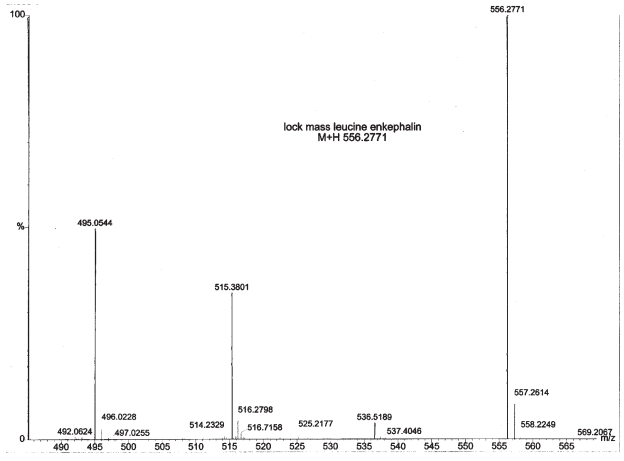

Figure S1. Electrospray mass spectrum of esculetin-4-carboxylic acid methyl ester (**1**).

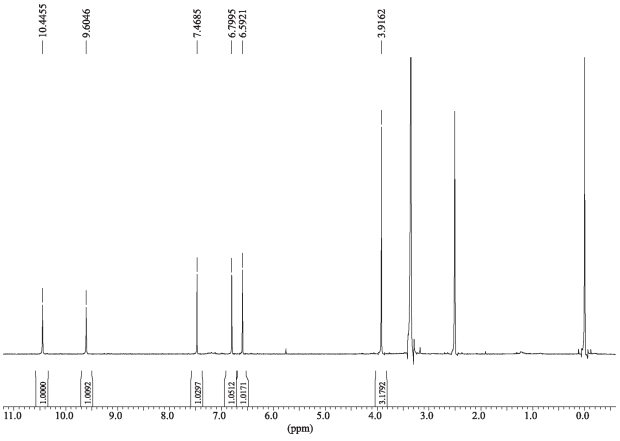

Figure S2. ^{1}H NMR spectrum of esculetin-4-carboxylic acid methyl ester (**1**) (DMSO-d_6, 400 MHz).

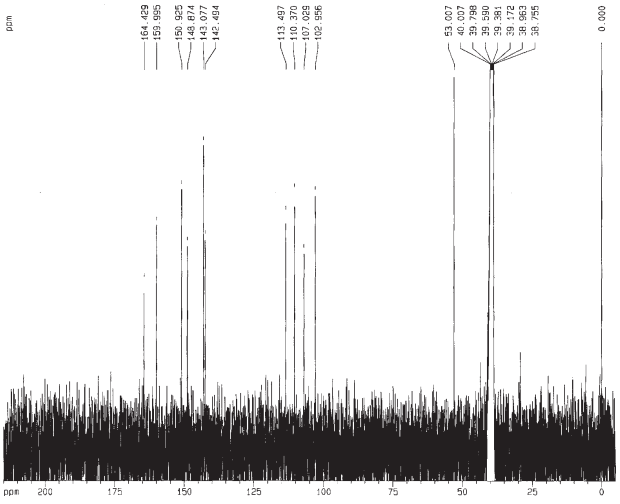

Figure S3. ^{13}C NMR spectrum of esculetin-4-carboxylic acid methyl ester (**1**) (DMSO-d_6, 100 MHz).

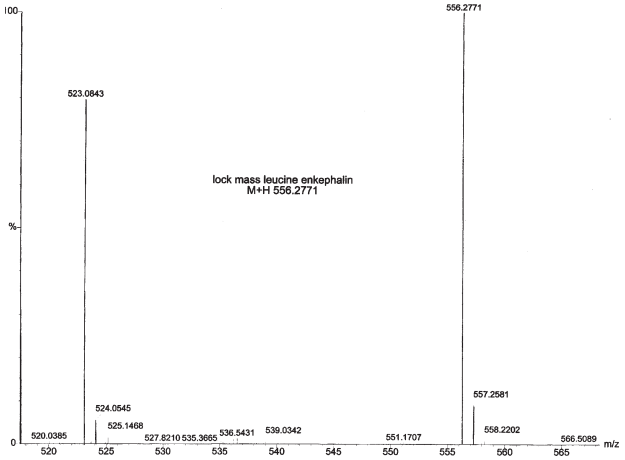

Figure S4. Electrospray mass spectrum of esculetin-4-carboxylic acid ethyl ester (**2**).

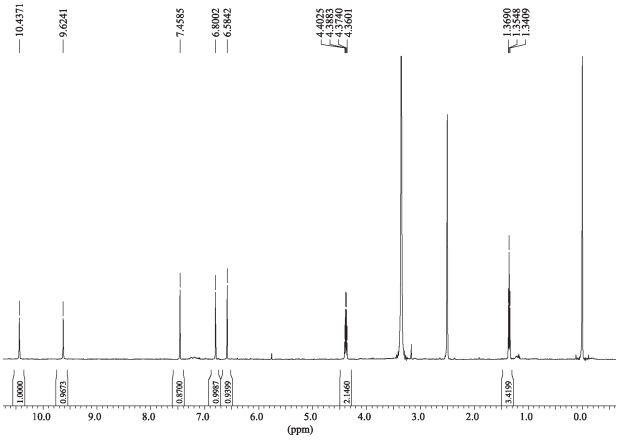

Figure S5. ^{1}H NMR spectrum of esculetin-4-carboxylic acid ethyl ester (**2**) (DMSO-d_6, 400 MHz).

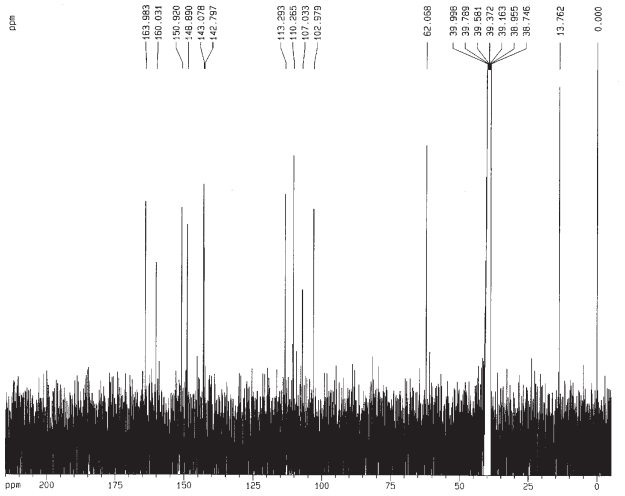

Figure S6. ^{13}C NMR spectrum of esculetin-4-carboxylic acid methyl ester (**2**) (DMSO-d_6, 100 MHz).