Synthesis of Essramycin and Comparison of Its Antibacterial Activity

Ernest H. L. Tee, Tomislav Karoli, Soumya Ramu, Johnny X. Huang, Mark S. Butler, and Matthew A. Cooper*

Institute for Molecular Bioscience, University of Queensland, St Lucia, Queensland 4072, Australia

Received September 15, 2010

The triazolopyrimidine natural product essramycin (**1**) was synthesized without the use of protecting groups via a two-step reaction scheme involving a 3-amino-1,2,4-triazole intermediate, and its structure was unequivocally determined. However, in contrast to the natural product, the synthetic essramycin (**1**) did not display any antibacterial activity.

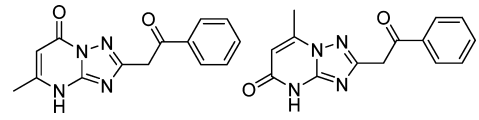
Essramycin (**1**) is a triazolopyrimidine that was isolated from a marine actinomycete (*Streptomyces* sp. Merv8102) obtained from sediment samples collected in the Egyptian Mediterranean Sea.[1] Spectroscopic data (IR, UV, HRMS, ^{1}H, ^{13}C, HSQC, and HMBC NMR) used in the initial structure elucidation of **1** could not unequivocally distinguish between the isomers **1** and **2** (Figure 1). However, comparison of the ^{13}C NMR chemical shifts with synthetic triazolopyrimidine analogues[2] allowed the structure of the isolated essramycin to be assigned as isomer **1**. Triazolopyrimidines display multifaceted biological properties, including antibacterial,[3,4] antimalarial,[5−8] leishmanicidal,[9] bronchodilatory,[10] vasodilatory,[11,12] anticancer,[13,14] immunosuppressive,[15] and anxiolytic[16] activities. Essramycin (**1**), which was claimed to be the first isolated triazolopyrimidine natural product, was reported to be active against *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Micrococcus luteus*.[1] The broad-spectrum antibacterial properties reported for **1** and the opportunity to explore a new antibacterial pharmacophore led us to undertake its synthesis. Herein, we present the total synthesis of **1** and the confirmation of its structure. We also report that the synthetically derived **1** displayed no antibacterial activity.

The synthesis of **1** (Scheme 1) proceeded via a 3-amino-1,2,4-triazole intermediate **3**, which was isolated as a minor product from the reaction between aminoguanidine and ethyl benzoylacetate.[17] Compound **3** and ethyl acetoacetate were then heated under acidic conditions[18] followed by recrystallization from methanol to yield compound **1** with 98% purity. The spectroscopic data of compound **1** were consistent with those previously reported.[1] The melting point of **1** was 22 °C higher than that reported for its naturally isolated counterpart, which most likely reflects the crystalline nature of the synthetic material against the amorphous nature of the natural product, which was isolated by column chromatography.

Analysis of the ^{1}H NMR (d_6-DMSO) of **1** and **3** revealed the presence of keto−enol tautomerism of the methylene protons adjacent to the ketone group.[19] Treatment of compound **1** with a tertiary base such as DIPEA (diisopropylethylamine) shifted the equilibrium in favor of the enol tautomer (from 6% to 20%), which assisted with the assignment of ^{1}H and ^{13}C NMR resonances of the enol form of **1** (Figure 2). Further evidence for the structure assignment of essramycin was obtained by analyzing the ^{1}H−^{15}N gHMBC NMR spectrum of **1** (Figure 3). Correlations between the methyl protons and the enaminone nitrogen (δ_N −253 ppm) and between the olefinic methine H-6 and the bridgehead hydrazine nitrogen (δ_N −155 ppm) unequivocally supported the structure assignment of essramycin as isomer **1**.

The antibacterial activity of synthetically derived **1** was evaluated against *S. aureus* (ATCC 25923), *E. faecalis* (ATCC 51299), *E. coli* (ATCC 25922), *K. pneumoniae* (ATCC 700603), *P. aeruginosa* (Meropenem-resistant), *A. baumannii* (Meropenem-resistant), and the strains used in the original study: *P. aeruginosa* (ATCC 10145) and *B. subtilis* (ATCC 6051).[1] However, no antibacterial activity was found for synthetically derived **1** at a maximum concentration of 64 μg/mL. Negative results were also obtained using the disk diffusion method and when **1** was assayed as the sodium salt and formulated with carboxymethylcellulose. To check whether **1** had been degraded or was only partially soluble during the antibacterial assays, its concentration in the assay plates was quantified by HPLC, and it was found to be present at an appropriate level. Furthermore, the binding of **1** to proteins in the LB assay broth (above 30 kDa) was determined by ultrafiltration to be only 7%. The kinetic solubility of **1** in water and pH 7.4 phosphate buffer was determined to be greater than 150 μg/mL, while its thermodynamic solubilities in water and pH 7.4 phosphate buffer were 175 and 898 μg/mL respectively. These data demonstrated that at a maximum concentration of 64 μg/mL the antibacterial results of **1** were not compromised by high protein binding and/or aqueous solubility limitations. Incidentally, **1** was not cytotoxic against HEK293 and HepG2 cell lines at a maximum concentration of 200 μM.

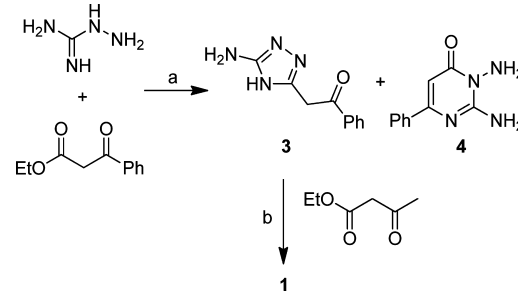
In conclusion, we have performed the total synthesis of essramycin (**1**) in two steps via a 3-amino-1,2,4-triazole intermediate. While the structure assignation was confirmed, the synthetically derived material did not possess any antibacterial activity as reported for the natural product.[1] After submission of this paper, Battaglia and Moody also published the synthesis of **1** using a similar synthetic methodology, confirming the structure assignation, but in the absence of associated biological data.[22] Unfortunately, the



1 **2**

Figure 1. Structure of essramycin (**1**) and its isomer (**2**).

Scheme 1. Synthesis of Essramycin (**1**)[a]



[a] Reagents and conditions: (a) *n*-butanol, reflux, 130 °C, 3 h, 5%; (b) glacial CH_3CO_2H, heat, 114 °C, 4 h, 18%.

* To whom correspondence should be addressed. Phone: +61 7 33462044. E-mail: m.cooper@uq.edu.au.

10.1021/np100648q
Published on Web 11/04/2010

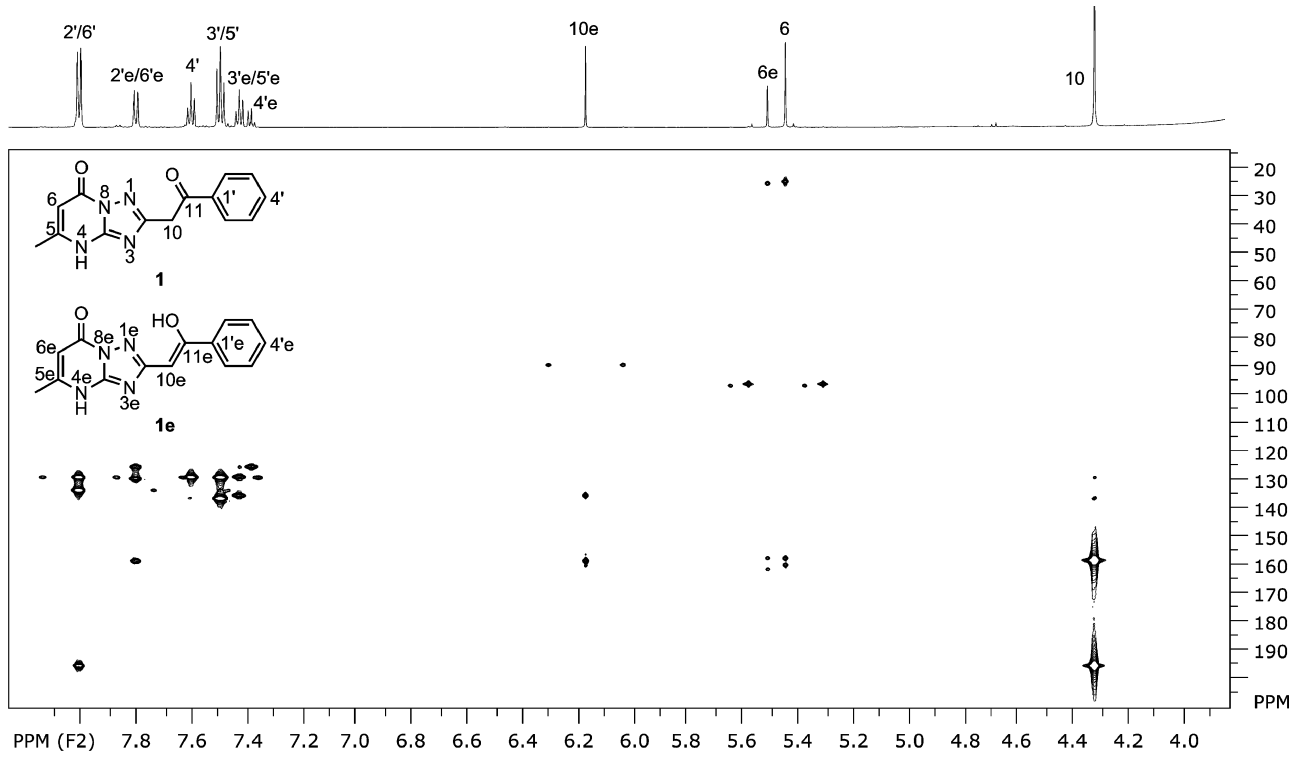


Figure 2. $^{1}H-^{13}C$ gHMBC spectrum of **1** and its enol tautomer (**1e**) after the addition of 2 molar equiv of DIPEA.

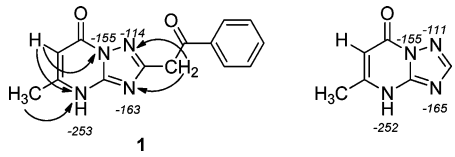


Figure 3. $^{1}H-^{15}N$ gHMBC correlations and ^{15}N chemical shifts for **1** (δ_N externally referenced to CH_3NO_2 at 0 ppm)[20,21] with published ^{15}N chemical shifts of the triazolopyrimidine core.[2]

originally naturally occurring material was not available for a direct comparison with the synthetic material. A recent review of the originally published experimental work leading to the first paper[1] suggests that the activity reported for the natural product was based upon testing of a crude sample, rather than purified material (personal communication, Prof. H. Laatsch). In our hands essramycin is not an antibiotic.

Experimental Section

General Experimental Procedures. NMR data were collected in d_6-DMSO at 298 K using either a Varian Unity 400 MHz or a Bruker Avance 600 MHz spectrometer as noted, and spectra calibrated to residual solvent signals and TMS. LC-MS was conducted using an Agilent 1200 system with UV, ELSD, and ESIMS detection (Agilent 6110 quadropole). HPLC columns used were Agilent Zorbax SB-C_{18} columns [(2.1 × 100 mm) or (9.4 × 100 mm)] and Agilent Zorbax Eclipse Plus Phenyl Hexyl columns (2.1 × 100 mm). Solvent A: CH_3CN with 0.1% (v/v) TFA; solvent B: H_2O with 0.1% (v/v) TFA; solvent C: CH_3CN with 0.05% (v/v) formic acid; solvent D: H_2O with 0.05% (v/v) formic acid. HPLC method 1: Agilent Zorbax SB-C_{18} (9.4 × 100 mm). One minute hold with A:B (2:98), linear gradient of A:B (2:98) to A:B (100:0) over 9 min, followed by 5 min hold with A:B (100:0). HPLC method 2: Agilent Zorbax Eclipse Plus Phenyl Hexyl column (2.1 × 100 mm). One minute hold with C:D (5:95), gradient of C:D (5:95) to C:D (100:0) over 8 min, followed by 2 min hold at C:D (100:0). IR spectra were scanned using a Perkin-Elmer Spectrum 2000 FTIR spectrometer as KBr disks, while UV spectra were obtained using a BMG Labtech PolarStar Omega microplate reader. The path length correction was determined by the microplate reader. High-resolution electrospray mass spectrometry (HRESMS) was performed on a Bruker MicrOTOF mass spectrometer, and the data obtained were analyzed on the Bruker DataAnalysis 4.0 software. The melting point determinations were measured using the Stuart SMP11 melting point apparatus, which was calibrated against benzoic acid. Thin-layer chromatography was performed on Merck silica gel 60 F_{254} plates and visualized using a UV lamp at 254 nm or 2% ninhydrin/ethanol. Flash chromatography employed the use of Merck silica gel 60, 40−63 μm. All commercial chemical reagents were used as received.

Synthesis of 2-(5-Amino-4*H*-1,2,4-triazol-3-yl)-1-phenylethanone (3).[17] A mixture of aminoguanidine bicarbonate (21.9073 g, 160.95 mmol), *n*-butanol (28 mL), and ethyl benzoylacetate (28 mL, 161.70 mmol) was stirred at 130 °C for 3 h under an inert nitrogen atmosphere. A dark brown solution was formed, which produced a yellowish-brown suspension upon cooling to room temperature. The product was collected by filtration, washed sequentially with ice-cold *n*-butanol (100 mL) and water (100 mL), and then dried in vacuo overnight (7.1 g, yield 22% with 54% purity at 254 nm by HPLC). The compound was further purified by stirring the product as a slurry in DMF (50 mL) at room temperature for 40 min. The resulting creamy white suspension was then filtered under vacuum to produce compound **3** as a white solid (1.78 g, 5% overall yield with 97% purity at 254 nm by HPLC). Further recrystallizations in DMF, THF, and ethanol did not increase the final product purity as analyzed by ^{1}H NMR and HPLC. Analysis of the ^{1}H NMR of **3** revealed keto−enol tautomerism in a 7:3 ratio. HPLC: t_R = 4.44 min using method 1; ^{1}H and ^{13}C NMR, see Supporting Information, Table S1; HRESMS (+ve) *m/z* 203.0922 (calcd for $C_{10}H_{11}N_4O$ $[M + H]^+$, 203.0927).

Synthesis of Essramycin (1). A solution of **3** (400 mg, 1.978 mmol), ethyl acetoacetate (250 μL, 1.975 mmol), and glacial acetic acid (1.2 mL) was stirred at 114 °C for 4 h, at which time a light brown precipitate had formed. Toluene was added, and the mixture was evaporated to dryness in vacuo (60 mbar, 40 °C water bath) to give a brown-white solid (517 mg, yield 98%). The product was recrystallized from methanol to give compound **1**, mp 241−243 °C (98% purity at 254 nm by HPLC) as colorless needles (96.4 mg, overall yield 18%). Further efforts to purify **1** by flash chromatography and preparative HPLC were unsuccessful: UV (MeOH) λ_{max} (log ε) 246 (4.68), 275 (4.50) nm; IR (KBr disk) ν_{max} 3061, 2934, 2818, 1684, 1660, 1606, 1568, 1518, 1426, 1375, 1219, 760, 692 cm^{-1}; full assignments of ^{1}H and ^{13}C NMR in Supporting Information, Table S3; HRESMS (+ve) *m/z* 269.1070 (calcd for $C_{14}H_{13}N_4O_2$ $[M + H]^+$, 269.1033), 559.1811 (calcd for $C_{28}H_{24}N_8O_4Na$ $[2M + Na]^+$, 559.1813).

Broth Dilution Antibacterial Assays. MIC assays were performed in triplicates on methicillin-susceptible *S. aureus* (ATCC 25923), *E. faecalis* VanB (ATCC 51299), *E. coli* (ATCC 25922), *K. pneumoniae* (ATCC 700603), and Meropenem-resistant *P. aeruginosa* and *A. baumannii* cultured in Luria−Bertani (LB) (Ambresco) and Mueller−Hinton (MH) broths. Overnight bacterial cultures in LB broth were diluted 40-fold into fresh LB and MH broths and shaken at 37 °C for 1 h. The resultant mid-log phase cultures ($OD_{600\ nm} \approx 0.6$) were diluted to a final concentration of 5×10^5 cfu/mL and were then added to assay wells containing 2-fold serial dilutions of the synthetic essramycin (**1**) from 64 to 0.03 μg/mL in a maximum DMSO concentration of 1.28%. Plates were incubated at 37 °C for 24 h, and the MIC was recorded as the lowest concentration of essramycin showing no visible bacterial growth. Tetracycline, vancomycin, and colistin were used as positive controls.

Disk Diffusion Antibacterial Assays. MIC assays were performed on methicillin-susceptible *S. aureus* (ATCC 25923), *E. faecalis* VanB (ATCC 51299), *E. coli* (ATCC 25922), *K. pneumoniae* (ATCC 700603), and Meropenem-resistant *P. aeruginosa* and *A. baumannii* cultured in LB broth. Bacterial cultures were prepared following the experimental procedures for the MIC determination of synthetic essramycin. The mid-log phase bacterial cultures ($OD_{600\ nm} \approx 0.6$) were streaked as a bacterial lawn on LB agar plates. Autoclaved Whatman filter paper discs were impregnated with 10 μL of 2-fold serial dilutions of essramycin from 32 to 1 μg/mL. The disks were placed onto the inoculated LB agar plates, and the zone of inhibition was measured after incubating the plates at 37 °C for 24 h. Tetracycline (64 μg/mL) was used as a positive control.

Essramycin Sodium Salt and Carboxymethylcellulose Formulation. Essramycin (**1**) was formulated with carboxymethylcellulose (CMC) by adding a 2% (w/v) aqueous CMC stock solution to an essramycin stock solution (4 mg/mL in DMSO) to give a final concentration of 1.28 mg/mL. The sodium salt of **1** was prepared by titrating the compound with NaOH (18.64 mM) to a point just after the buffer region. MIC assays were performed in triplicates on LB broth cultures of methicillin-susceptible *S. aureus* (ATCC 25923), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 10145), and *B. subtilis* (ATCC 6051) following the experimental protocols for the MIC determination of synthetic **1**.

Acknowledgment. Financial support for this project was provided by the National Health and Medical Research Council (NHMRC) grant AF 511105.

Supporting Information Available: NMR data and spectra of **3**, **1**, and **1** with DIPEA, comparison of the physicochemical and NMR data of the naturally occurring and synthetic essramycin, protein-binding assay, solubility determination, and cytotoxicity assay. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) El-Gendy, M. M. A.; Shaaban, M.; Shaaban, K. A.; El-Bondkly, A. M.; Laatsch, H. *J. Antibiot.* **2008**, *61*, 149–157.
(2) Kleinpeter, E.; Thomas, St.; Fischer, G. *J. Mol. Struct.* **1995**, *355*, 273–285.
(3) Shaaban, M. R. *Heterocycles* **2008**, *75*, 3005–3014.
(4) Shaaban, M. R.; Saleh, T. S.; Farag, A. M. *Heterocycles* **2007**, *71*, 1765–1777.
(5) Deng, X.; Gujjar, R.; El Mazouni, F.; Kaminsky, W.; Malmquist, N. A.; Goldsmith, E. J.; Rathod, P. K.; Phillips, M. A. *J. Biol. Chem.* **2009**, *284*, 26999–27009.
(6) Gujjar, R.; Marwaha, A.; El Mazouni, F.; White, J.; White, K. L.; Creason, S.; Shackleford, D. M.; Baldwin, J.; Charman, W. N.; Buckner, F. S.; Charman, S.; Rathod, P. K.; Phillips, M. A. *J. Med. Chem.* **2009**, *52*, 1864–1872.
(7) Phillips, M. A.; Gujjar, R.; Malmquist, N. A.; White, J.; El Mazouni, F.; Baldwin, J.; Rathod, P. K. *J. Med. Chem.* **2008**, *51*, 3649–3653.
(8) Werbel, L. M.; Elslager, E. F.; Chu, V. P. *J. Heterocycl. Chem.* **1973**, *10*, 631–635.
(9) Ram, V. J.; Srivastava, P.; Singh, S. K.; Kandpal, M.; Tekwani, B. L. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1087–1090.
(10) Davies, G. E. *J. Pharm. Pharmacol.* **1973**, *25*, 681–689.
(11) Pfeifer, S.; Wierer, A.; Rohde, J.; Mann, I. *Pharmazie* **1971**, *26*, 539–548.
(12) Sato, Y.; Shimoji, Y.; Fujita, H.; Nishino, H.; Mizuno, H.; Kobayashi, S.; Kumakura, S. *J. Med. Chem.* **1980**, *23*, 927–937.
(13) Richardson, C. M.; Williamson, D. S.; Parratt, M. J.; Borgognoni, J.; Cansfield, A. D.; Dokurno, P.; Francis, G. L.; Howes, R.; Moore, J. D.; Murray, J. B.; Robertson, A.; Surgenor, A. E.; Torrance, C. J. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 1353–1357.
(14) Zhang, N.; Ayral-Kaloustian, S.; Nguyen, T.; Afragola, J.; Hernandez, R.; Lucas, J.; Gibbons, J.; Beyer, C. *J. Med. Chem.* **2007**, *50*, 319–327.
(15) Saiga, K.; Toyoda, E.; Tokunaka, K.; Masuda, A.; Matsumoto, S.; Mashiba, H.; Kuramochi, H.; Nemoto, K.; Abe, F.; Kawagishi, N.; Furukawa, H.; Ono, M. *Bone Marrow Transplant.* **2006**, *37*, 317–323.
(16) Dusza, J. P.; Albright, J. D. U.S. Patent 4,444,774, 1984.
(17) Hlavka, J. J.; Bitha, P.; Lin, Y.; Strohmeyer, T. *J. Heterocycl. Chem.* **1984**, *21*, 1537–1541.
(18) Kofman, T. P.; Uvarova, T. A.; Kartseva, G. Y.; Uspenskaya, T. L. *Russ. J. Org. Chem.* **1997**, *33*, 1784–1793.
(19) Kolehmainen, E.; Osmialowski, B.; Nissinen, M.; Kauppinen, R.; Gawinecki, R. *J. Chem. Soc., Perkin Trans. 2* **2000**, 2185–2191.
(20) Harris, R. K.; Becker, E. D.; de Menezes, S. M. C.; Goodfellow, R.; Granger, P. *Pure Appl. Chem.* **2001**, *73*, 1795–1818.
(21) Martin, G. E.; Hadden, C. E. *J. Nat. Prod.* **2000**, *63*, 543–585.
(22) Battaglia, U.; Moody, C. J. *J. Nat. Prod.* **2010**, *73*, in press.

NP100648Q