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Streptopyrrole: an Antimicrobial Metabolite from *Streptomyces armeniacus*

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A colourless, crystalline metabolite, $C_{14}H_{12}ClNO_4$, named streptopyrrole, has been isolated from submerged fermentation cultures of *Streptomyces armeniacus* by extraction, followed by chromatographic purification. Its tricyclic molecular framework, seemingly without natural product precedents, as well as its substitution pattern were derived from extensive NMR analysis including ^{1}H-detected INADEQUATE experiments. Streptopyrrole exhibits weak growth inhibitory effect towards a broad range of microorganisms.

Species of the microbial class Actinomyces, especially *Streptomyces* strains, are well known sources of a multitude of structurally diverse metabolites, several of which possess interesting and important properties. Hence, it was appropriate to include *S.* species in our search for microbial metabolites with growth inhibitory effects against phytopathogenic fungi. We report the isolation, structure elucidation, and biological properties of *streptopyrrole*, a crystalline metabolite produced by *S. armeniacus* and possessing a tricyclic structure, seemingly unprecedented within the class of natural products. Efficient use was made of various, recently developed pulsed field gradient assisted NMR techniques in the structure elucidation.

Isolation, properties and structure elucidation

Submerged fermentations of a *S. armeniacus* strain (ATCC 15676), solvent extraction, chromatographic purification, and crystallization, afforded streptopyrrole (about 15 mg per litre of culture broth) as colourless needles with the molecular composition $C_{14}H_{12}ClNO_4$, established by elemental analysis and HR-EIMS and signifying an index of hydrogen deficiency of nine.

The ^{1}H NMR spectrum of streptopyrrole (Table 1) displayed signals attributable to a propyl group, one singlet plus two mutually coupled (J=2 Hz) aromatic protons, and two exchangeable phenolic protons, one of which appeared as a sharp singlet at low field (δ 11.01) and hence obviously hydrogen-bonded to a carbonyl grouping. The presence of the latter was consistent with a strong absorption band at 1640 cm^{-1} in the IR-spectrum. These data, combined with the molecular composition, are interpretable only in terms of a tricyclic structure of streptopyrrole. The ^{13}C NMR spectrum (Table 1) of streptopyrrole displayed signals corresponding to 14 non-equivalent carbon atoms. 2D Measurements (HSQC and HMBC) were helpful in assigning proton-bearing carbon atoms and identifying several ^{1}H–^{13}C long-range correlations, respectively, but insufficient for unambiguous structure establishment, chiefly due to the difficulty of distinguishing between

Table 1. ^{1}H and ^{13}C NMR data for streptopyrrole (**1**) in DMSO-d_6.

C[a]	^{13}C	^{1}H
1	89.9[b]	6.02 (d, J=2.0 Hz)
2	116.8	
3	104.0[c]	7.23 (d, J=2.0 Hz)
5	157.8	
5a	91.9	
6	158.8	
7	111.2	
8	163.6	
9	93.2	6.33 (s)
9a	152.7	
10a	140.5	
11	23.6	2.47 (t, J=7 Hz)
12	21.3	1.46 (sextet, J=7 Hz)
13	13.8	0.89 (t, J=7 Hz)

[a]Numbering according to formula (**1**). [b]$^1J_{CH}$=185 Hz. [c]$^1J_{CH}$=202 Hz.

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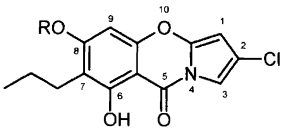
two-, three-, or even four-bond correlations. Help was therefore sought, and found, in the recently developed, pulsed field gradient assisted NMR techniques, INEPT2–INADEQUATE, HMBC–INADEQUATE, and 2Q HMBC.[1–3] The first of these proton-detected ^{13}C–^{13}C correlation techniques discloses coupling between pairs of carbon atoms provided at least one pair-member is hydrogen-bearing, whereas the second reveals *J*-coupled carbon atoms of which at least one exhibits ^{1}H–^{13}C long-range coupling. Both techniques are applicable in $^{1}J_{CC}$- or $^{n}J_{CC}(n>1)$-versions, optimized for detection of one-bond and long-range ^{13}C–^{13}C couplings, respectively. The 2Q HMBC-technique, finally, operates in a J_{CC}-independent manner and is capable of disclosing correlations within carbon atom pairs provided each pair-member exhibits ^{1}H–^{13}C long-range coupling. Taking advantage of the abovementioned techniques the structure of streptopyrrole was unambiguously established as 2-chloro-6,8-dihydroxy-7-propyl-5*H*-pyrrolo[2,1-*b*]-[1,3]-benzoxazin-5-one (**1**).

Discrimination between the ten-carbon and the four-carbon pyrrole moieties of streptopyrrole (**1**) became feasible by detections at H-1, H-3, and H-9 of both vicinal and long-range ^{13}C–^{13}C correlations in the INEPT2–INADEQUATE spectra (Fig. 1), corroborated by the $^{1}J_{CC}$-HMBC–INADEQUATE spectrum (Fig. 2) [including its $^{n}J_{CC}$-version (not shown)] and schematically shown in Fig. 3. Table 2 summarizes all ^{13}C–^{13}C correlations established by the INADEQUATE techniques specified above. The structure elucidation of streptopyrrole serves well as a typical case where condensed, proton-deficient aromatic rings, sparse in ^{1}H–^{1}H couplings but abundant in ^{1}H–^{13}C long-range correlations of questionable diagnostic value, make unequivocal structure assignment by standard NMR-techniques difficult or impossible, but straightforward with the new techniques. Even so, it should be noted that however successful the $^{1}J_{CC}$-versions of INEPT2 and HMBC–INADEQUATE techniques are in establishing ^{13}C–^{13}C connections involving carbon atoms separated from the nearest proton by up to four bonds, the corresponding $^{n}J_{CC}$-versions, principally allowing the disclosure of more remote connectivities, must be treated with caution inasmuch as two-, three-, and four-bond ^{13}C–^{13}C couplings may be similar in magnitude and hence of limited diagnostic value in structural assignments.

Additional features supporting the structure of streptopyrrole (**1**) include characteristically large, pyrrole-ring based $^{1}J_{C1,H}$- and $^{1}J_{C3,H}$-constants (185 and 202 Hz, respectively), and the high-field ^{13}C signal (δ 158.8) corresponding to the C5-carbonyl function, indicative of its hydrogen-bonding to the low-field HO-proton at C6.



1, R = H
2, R = CH_3

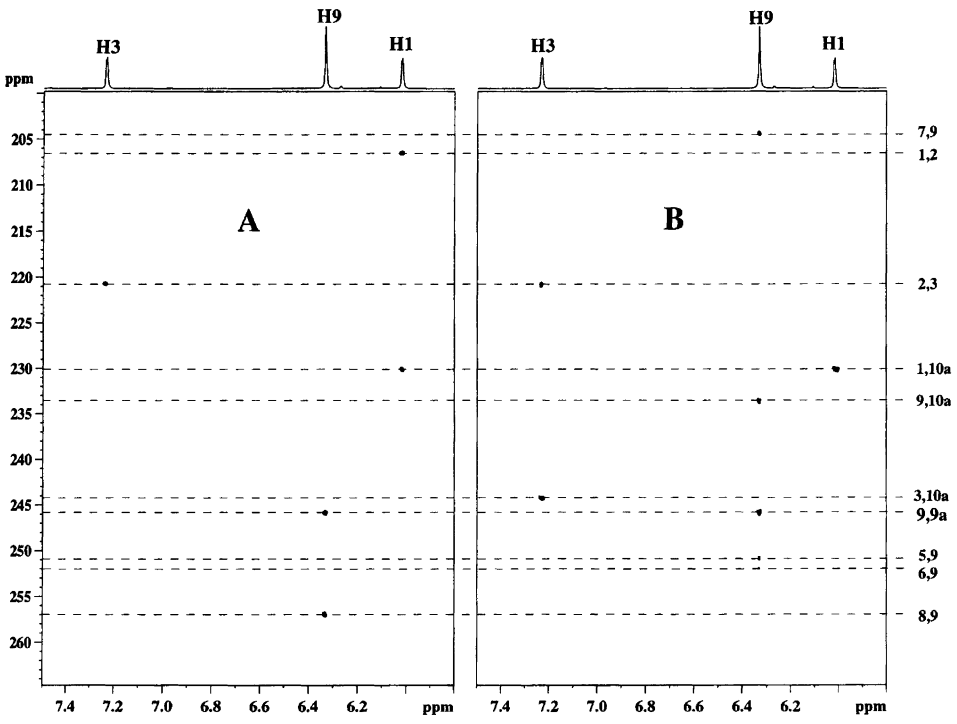


Fig. 1. Sections of the $^{1}J_{CC}$-INEPT2- ($^{1}J_{CC}=60$ Hz) (**A**) and $^{n}J_{CC}$-INEPT2–INADEQUATE ($^{n}J_{CC}=8$ Hz) (**B**) spectra of streptopyrrole (**1**). Spectrum **A** exhibits signals attributable to one-bond ^{13}C–^{13}C couplings only, whereas in **B** both one-, two-, and three-bond correlations are observed.

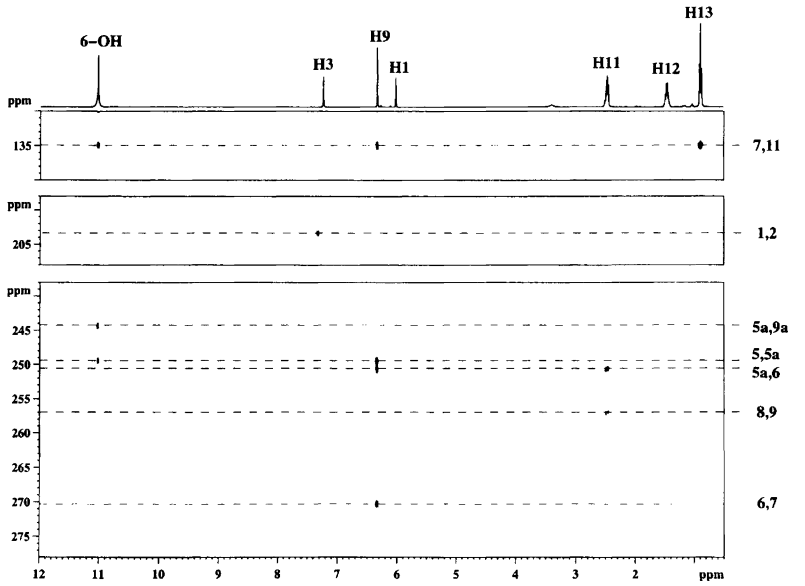


Fig. 2. Slices of the $^{1}J_{CC}$-HMBC–INADEQUATE ($^{1}J_{CC}$ = 55 Hz) spectrum of streptopyrrole (**1**).

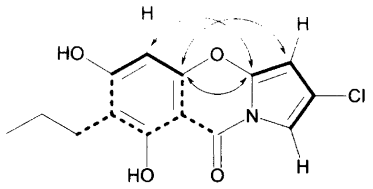
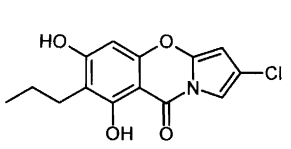
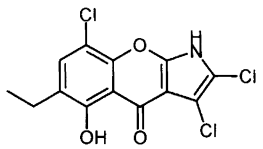


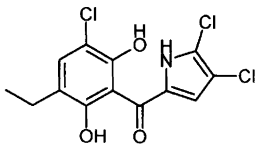
Fig. 3. One-bond $^{1}J_{CC}$-connections in streptopyrrole (**1**) established by $^{1}J_{CC}$-INEPT2- (solid lines) and $^{1}J_{CC}$-HMBC–INADEQUATE (broken lines) experiments. Arrows signal diagnostic long-range ^{13}C–^{13}C correlations observed in $^{n}J_{CC}$-optimized versions of the experiments.



1



3



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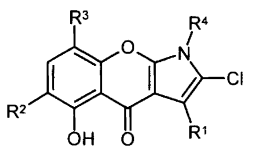

5

Upon treatment with diazomethane streptopyrrole was converted into a mono-*O*-methyl derivative (**2**) structurally identified by its exchangeable low-field HO-proton and the HMBC-correlation between the methoxy-group protons and C8, established by NMR techniques.

Table 2. Correlations observed in HMBC, INEPT2–INADEQUATE, HMBC–INADEQUATE, and 2Q HMBC spectra of streptopyrrole (**1**).

H[a]	^{1}H (δ)	HMBC J_{CH} = 7 Hz	INEPT2–INAD J_{CC} = 60 Hz	INEPT2–INAD[b] J_{CC} = 8 Hz	HMBC–INAD J_{CC} = 55 Hz	HMBC–INAD[b] J_{CC} = 8 Hz	2Q HMBC
H1	6.02	C3, C5, C10a	C1–C2, C1–C10a	C1–C3			
H3	7.23	C1, C2, C5, C10a	C2–C3	C1–C3, C3–C10a	C1–C2		
H9	6.33	C5, C5a, C7, C8, C9a	C8–C9, C9–C9a	C5–C9, C6–C9 C7–C9, C9–C10a	C5–C5a, C5a–C6, C6–C7, C7–C8,[c] C7–C11	C1–C9a,[d] C2–C5, C2–C9a/C5–C7, C5–C6/C8–C9a, C5a–10a, C6–C8, C6–C9a, C9a–C10a	C5–C5a, C5–C9a, C5a–C7, C5a–C8, C5a–C9a, C7–C8, C7–C9a, C8–C9a
H11	2.47	C6, C7, C8, C12			C5a–C6, C8–C9		
H13	0.89	C11, C12			C7–C11		
6-OH	11.01	C5a, C6, C7			C5–C5a, C5a–C9a, C6–C7, C7–C8,[c] C7–C11		

[a]Numbering according to formula (**1**). [b]One-bond correlations not listed. [c]Observed in experiment optimized for J_{CC} = 45 Hz. [d]Observed in experiment optimized for J_{CC} = 4 Hz.

Biological activity and discussion

Streptopyrrole, when tested by agar diffusion assays, was found to exhibit weak growth inhibitory activity against a broad range of fungi, including species of Ascomycota, imperfect fungi, yeasts, and bacteria.

Though apparently lacking direct precedents as a natural product moiety the pyrrolo[2,1-*b*]--5*H*-[1,3]benzoxazin-5-one ring system of streptopyrrole (**1**) is not without close structural counterparts within the plethora of microbial metabolites. Thus, the co-occurring, Actinomycetes-derived antibiotics TAN-876A (**3**) and TAN-876B (**4**),[4] as well as a class of seven antibiotics, generically known as pyralomicins (**5**) (R^1, R^2, and R^3 = H and/or CH_3 and/or Cl),[5–7] produced by *Microtetraspora spiralis* (Actinomycetes), are all molecules probably possessing more than accidental structural resemblance to streptopyrrole (**1**). Biosynthetic studies have ascertained that the 3-carbonyl pyrrole unit of the major member of the pyralomicin family (**5**) derives from a (rearranged) proline unit.[7] Again, though experimentally unproven, **3** may have a similar origin whereas the bicyclic congener (**4**) may, by inspection, be regarded as a straightforward proline derivative.[7] In this light proline may also conceivably be the source of the pyrrole *N*-carbonyl moiety of streptopyrrole (**1**), an intriguing though yet unproven possibility.

Experimental

^1H and ^{13}C NMR data were acquired at 300 K on a Bruker DRX400 instrument. Chemical shifts (δ), measured for samples in DMSO-d_6 and chloroform-*d*, are in ppm relative to TMS, coupling constants (*J*) in Hz. The ^1H-detected INADEQUATE-experiments were acquired on a 0.34 M sample of streptopyrrole (**1**) in DMSO-d_6 using pulse sequences essentially as described by Meissner *et al.*[3] Measuring times ranged from 20 to 48 h. EI mass spectra, at 70 eV ionization potential, were recorded on a JEOL AX505W instrument, and are presented as *m/z* (% rel. int.). Antimicrobial activity was assessed in agar diffusion assays as previously described.[8]

Cultivation and submerged fermentation. A strain of *Streptomyces armeniacus* (ATCC 15676) was propagated on oatmeal agar (OMA) slants (12 ml/slant) for 6 days at 30 °C. The OMA was prepared by mixing oatmeal (20 g), yeast extract (2.5 g), and water (*ad* 1 l). This mixture was brought to the boiling point and then stirred at 60 °C for 4 h. Agar (Merck, 20 g) was added and the mixture again brought to the boiling point. Finally, the medium was autoclaved at 121 °C for 20 min. The submerged fermentation was conducted in 250 ml baffled shake flasks each containing 100 ml of growth medium. The latter was prepared by mixing yeast extract (4 g), soluble starch (15 g), K_2HPO_4 (1 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), and distilled water (*ad* 1 l), and then autoclaved for 20 min at 121 °C. Shake flasks were inoculated by a suspension (5 ml per flask) prepared by adding sterile water (10 ml) containing 0.1% Tween to OMA slants. Fermentation was carried out for 7 days at 30 °C and 250 rpm.

Extraction and isolation of streptopyrrole. Biomass from 20 l of culture broth was harvested by filtration after addition of Celite (2%). The filter cake was extracted with three 3.5 l portions of MeOH with stirring. The solvent was evaporated off, water (3.5 l) was added, and the resulting aqueous suspension extracted four times with 3 l portions of EtOAc. Evaporation of the combined organic extracts yielded an aqueous residue, which was lyophilized to yield a brownish oil (7.9 g), to which were added heptane (450 ml), EtOAc (50 ml), MeOH (450 ml), and water (50 ml). After thorough mixing, followed by phase separation, the lower, aqueous phase was freed of solvent by evaporation to yield an oily residue (4.1 g). The residue from the solvent partition, divided into five portions, was applied to a silica gel column [Si60 40–63 µm, Lobar size B (Merck)], and eluted at a flow rate of 7.5 ml min^{-1} with a heptane–EtOAc gradient [90:10 → 90:10 (10 min) → 0:100 (45 min)]. The effluent was monitored by UV-absorption at 295 nm. The fractions containing streptopyrrole were pooled and the solvent evaporated off. The residue was dissolved in EtOAc (5 ml) and, following addition of heptane, streptopyrrole precipitated as an amorphous, buff solid (277 mg). Two recrystallizations from acetonitrile afforded streptopyrrole (**1**) as colourless needles, m.p. 181–183 °C (decomp.). Anal. $C_{14}H_{12}ClNO_4$: CHNCl; ^1H and ^{13}C NMR: see Table 1. HR-EIMS: found: 293.0457 (M^+), calc. for $C_{14}H_{12}{}^{35}ClNO_4$ 293.0455, found: 264.0063 $(M-Et)^+$, calc. for $C_{12}H_7{}^{35}ClNO_4$ 264.0063. EIMS: 295 (13), 293 (37), 266 (33), 264 (100), 149 (6), 138 (8), 94 (6), 40 (10). IR (KBr): 2950, 2928, 1640, 1584, 1514, 1467, 1297, 1193, 1120, and 1100 cm^{-1}. UVλ_{max}/nm (log ε) (MeOH): 241 (4.51), 294 (4.30), 335 (3.65).

Methylation of streptopyrrole. Streptopyrrole (4 mg) was treated with excess diazomethane in Et_2O containing catalytic amounts of silica gel. Preparative TLC (Merck Si60, heptane–EtOAc 2:1) yielded the mono-*O*-methyl ether (**2**) (2.3 mg) as a colourless solid. ^1H NMR ($CDCl_3$): δ 11.1 (1 H, s, 6-OH), 7.19 (1 H, d, *J*=2, H-3), 6.35 (1 H, s, H-9), 5.84 (1 H, d, *J*=2, H-1), 3.91 (3 H, s, 8-OCH_3), 2.63 (2 H, t, *J*=7, H_2-11), 1.53 (2 H, m, H_2-12) and 0.95 (3 H, t, *J*=7, H_3-13). ^{13}C NMR: δ 103.9 (d, C-3, $^1J_{CH}$=208 Hz), 118.5 (s, C-2), 90.4 (d, C-1), 140.8 (s, C-10a), 153.8 (s, C-9a), 89.7 (d, C-9), 164.9 (s, C-8), 113.7 (s, C-7), 159.0 (s, C-6), 93.5 (s, C-5a), 158.4 (s, C-5), 25.1 (t, C-11), 21.9 (t, C-12), 10.0 (q, C-13) and 56.0 (q, 8-OCH_3). EIMS: 309 (13), 307 (37), 292 (13), 280 (33), 278 (100), 248 (20), and 138 (7).

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