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Helquinoline, a New Tetrahydroquinoline Antibiotic from *Janibacter limosus* Hel 1[†]

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The ethyl acetate extract of cultures of *Janibacter limosus* showed a high biological activity against bacteria, and fungi and delivered two new natural products, a tetrahydroquinoline derivative designated as helquinoline (**1**), and the *N*-acetylkynuramine (**3a**), along with other known secondary metabolites. The structure of **1** has been elucidated as 4-methoxy-2-methyl-1,2,3,4-tetrahydroquinoline-8-carboxylic acid on the basis of 1D and 2D NMR and mass spectra. The relative stereochemistry of the compound **1** was assigned as 2R*,4R* with the aid of coupling constants, NOESY correlation and by comparison with a related compound.

Tetrahydroquinoline derivatives containing simple or complex substituents are used as pharmaceutical agents, pesticides, antioxidants, and corrosion inhibitors[1]. Among them, oxamniquine, a schistosomicide[2], nicainoprol, an antiarrhytmic drug[3], and virantmycin, a novel antiviral antibiotic[4], are well known. The synthetic tetrahydroquinoline L-689,560 (**2**) is one of the most potent NMDA antagonists[5] so far known in the literature. Due to their medical importance, varieties of tetrahydroquinoline derivatives have been synthesized by different methods[6]. The information pertaining to the preparation and medical application of these structurally related tetrahydroquinoline derivatives have appeared, however, more often in the patent literature than in the scientific journals[7].

In the course of our screening program for novel bioactive compounds from microorganisms, the ethyl acetate extract of the North Sea bacterium isolate Hel-1 drew our attention due to a nonpolar colourless zone with a strong blue fluorescence under 366 nm similar to anthranilic acid. Other than the latter, the zone did not give any positive reaction with anisaldehyde/sulfuric acid or Ehrlich's reagent. In addition, the high biological activity against *Streptomyces viridochromogenes*, *Staphylococcus aureus*, *Mucor miehei*, *Chlorella vulgaris*, *Chlorella sorokiniana*, and *Scenedesmus subspicatus* deserved further interest. Hel-1 was identified as a strain of *Janibacter limosus* (99% identity of the complete sequence), a rare group of Gram-positive strains, described 1997 by RAINEY *et al.*[8].

Working up of the extract guided by biological activity and chemical screening resulted in the isolation of **1** and **3a** as new natural products, together with several known compounds, anthranilic acid[9], *cyclo*(isoleucylprolyl)[10], *cyclo*(tyrosylprolyl)[11], indole-3-carbaldehyde[12], 2-(*p*-hydroxyphenyl)ethanol[13], phenylacetamide[14], indole-3-carboxylic acid[15], N_β-acetyltryptamine (**4a**)[16], *N*-acetyl-α-oxotryptamine (**4b**)[17], *p*-hydroxyphenylpropanoic acid[18], crotonic acid, and thymine. In this paper, we report the structure elucidation of **1** and **3a** as well as the biological activities.

Fermentation and Isolation

The isolate *Janibacter limosus* Hel 1 was initially grown in Erlenmeyer flasks (100 ml and 500 ml scale) at 27°C and

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100 rpm and then transferred to a 50 liters bioreactor. The working volume of the latter was 40 liters, the ingredients consisted of artificial seawater, Luria-Bertani broth and 50 g/liter glucose with an initial pH 7.2. After 48 hours of incubation at 27°C and 200 rpm, the aerobic batch fermentation was stopped. The end parameters were as follows: pH 4.5, 11 g/liter biomass, 15 g/liter residual glucose and totally 4.62 g of organic extract from 40 liters culture. The crude extract was dissolved in methanol and defatted by shaking with cyclohexane.

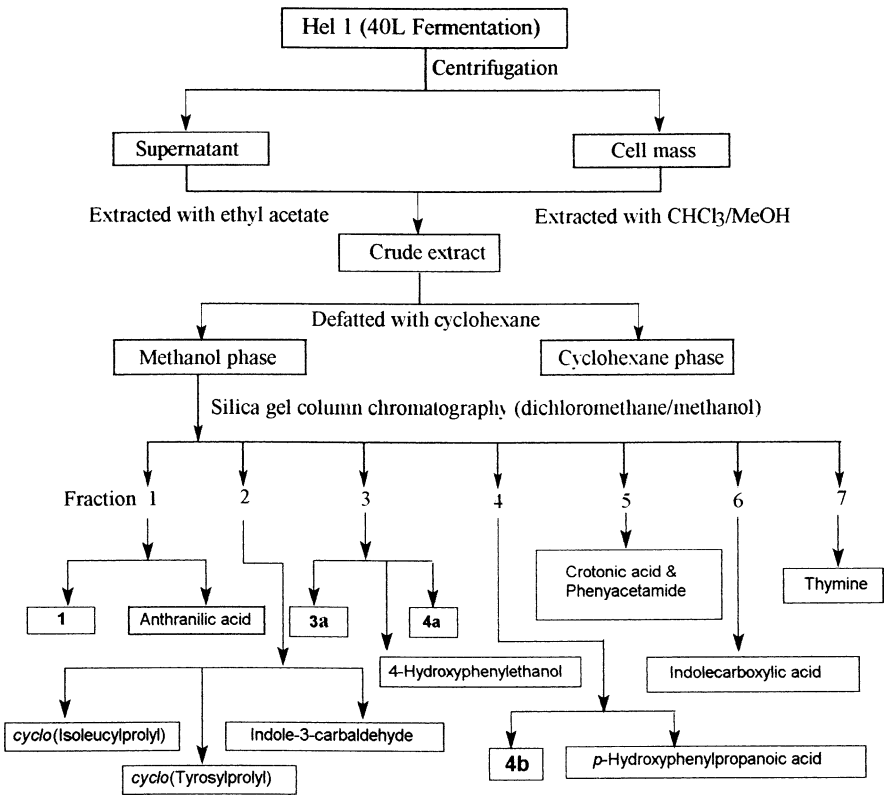
A bio-activity guided fractionation of the defatted extract (flash silica gel column, dichloromethane/methanol gradient) delivered four active fractions 1, 2, 3 and 4 (see Fig. 1). Fraction 1 was further purified by chromatography on Sephadex LH 20 and PTLC to afford helquinoline (**1**), along with anthranilic acid. The structure of compound **1** has been assigned by a detailed interpretation of ^{1}H, ^{13}C, H,H-COSY, HMQC, HMBC, NOESY, EI, and ESI mass spectra. On purification by HPLC, fraction 2 afforded cyclo(isoleucylprolyl), cyclo(tyrosylprolyl), and indole-3-carbaldehyde, while fraction 3 by purification on Sephadex LH-20 followed by HPLC gave *N*-acetylkynuramine (**3a**) along with *p*-hydroxyphenylethanol, and N_{β}-acetyltryptamine (**4a**). Fraction 4 yielded *N*-acetyl-α-oxotryptamine (**4b**), and *p*-hydroxyphenylpropanoic acid. Phenylacetamide and crotonic acid were obtained as a mixture from fraction 5. Two UV absorbing bands in fractions 6 and 7 yielded the frequently occurring derivatives indole-3-carboxylic acid and thymine. The known compounds were identified by comparison with data from AntiBase[19].

Results and Discussion

Compound **1** was obtained from fraction 1 as a colourless oil. The (+)- and (−)-ESI mass spectra showed *quasi* molecular ion peaks at *m/z* 244.1 ($[M+Na]^{+}$) and 220.4 ($[M-H]^{-}$, 463.5 ($[2M-2H+Na]^{-}$), respectively, corresponding to a molecular weight of 221 Dalton. The EI mass spectrum showed a molecular ion peak at *m/z* 221.1052 (HR-EIMS), corresponding to a formula of $C_{12}H_{15}O_3N$.

The ^{1}H NMR spectrum showed a doublet at δ 1.34 (3H, J=6.6 Hz) of a CH-CH_3 fragment, a triplet of a doublet at δ 1.48 (1H, J=13.5 and 2.7 Hz) and a doublet of a triplet at δ 2.14 (1H, J=13.5 and 3.0 Hz) of a methylene group. Furthermore, a multiplet at δ 3.74, a triplet at δ 4.20 with

Fig. 1. Work-up scheme of the strain Hel-1.



intensity of 1 proton each and a methoxy signal at δ 3.38 were observed. A broad signal at δ 10.5 was assigned to an NH proton. The three signals at δ 6.54 (t, J=8.1 Hz), 7.22 (dd, J=8.1 and 1.8 Hz), and 7.92 (dd, J=8.1 and 1.8 Hz), with a relative intensity of one proton each were indicative for a 1,2,3-trisubstituted benzene derivative.

The ^{13}C NMR spectrum disclosed 12 carbon atoms which were assigned on the basis of HMQC and H,H COSY data as one methyl, one methoxy, one methylene group, five methine and four quaternary carbon atoms. The signal at δ 173.5 suggested the presence of either a carboxylic acid or an ester.

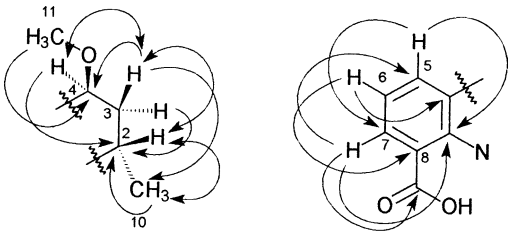
The H,H COSY spectrum suggested the presence of a CH_3–CH–CH_2–CH–O fragment (A) which was further confirmed by the HMBC spectrum. Similarly, the 3-substituted anthranilic acid fragment B was constructed (Fig. 2).

As the proton signal at δ 4.2 in fragment A showed a three bond coupling with C-5 and C-8a in fragment B, while the methyl signal at δ 1.34 showed a four bond coupling with C-8a, the fragments A and B must overlap, resulting in structure **1**.

The relative stereochemistry at the two chiral centres was assigned by the coupling patterns of the protons at C-2, C-3 and C-4, and by NOESY experiments. The proton at C-2 gave only a multiplet with coupling constants of J<10 Hz, indicating that it is in an equatorial position. H-4 showed a triplet of J=3 Hz due to a coupling with CH_2-3 from a *quasi*-equatorial position as well.

In the NOESY spectrum, H-4 (δ 4.2) showed a strong cross-peak with H-5 (δ 7.22), confirming this conformation where H-4 is in equatorial and the methoxy group in an axial position. As there was no correlation between the methoxy (δ 3.38) and the methyl (δ 3.72) group, both must be in *trans* orientation of a boat-shaped ring. Thus, the relative configuration at C-2 and C-4 in compound **1** was assigned as 2R*,4R*. This was confirmed by comparison

Fig. 2. Partial structures **A** and **B** of compound **1** derived from 2D NMR measurements (H,H-COSY (↔) and HMBC (→) couplings).



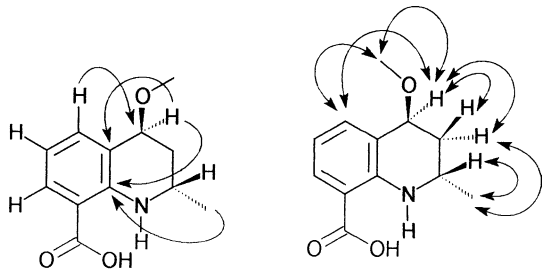
A **B**

Table 1. Physico-chemical properties of helquinoline (**1**) and *N*-acetylkynuramine (**3a**).

	Helquinoline (**1**)	N-Acetylkynuramine (**3a**)
Appearance	Colourless oil	Light yellow oil
R_f	0.55[a]	0.52[b]
Molecular formula	$C_{12}H_{15}O_3N$	$C_{11}H_{14}O_2N_2$
HREIMS exp.	221.1052	206.1055
calcd.	221.10519	206.10552
(+)-ESI-MS	244.1 ($[M+Na]^+$)	207.3 ($[M+H]^+$), 229.4 ($[M+Na]^+$), 435 ($[2M+Na]^+$)
(-)-ESI-MS	220.4 ($[M - H]^-$), 463.5 ([2M – 2H + Na])	
UV(MeOH) λ_{max}	349 (3.49), 261 (3.67), 221 (4.05)	362 (3.61), 227 (4.31), 202 (4.21)
UV(MeOH+HCl) λ_{max}	358 (3.47), 261 (3.59), 223 (4.03), 201 (3.96)	363 (3.18), 231 (4.05), 203 (4.13)
UV(MeOH+NaOH) λ_{max}	344 (3.43), 262 (3.69), 213 (4.05)	360 (3.68), 255 (3.77), 227 (4.31), 208 (4.30)
IR (KBr) ν cm^{-1}	3362, 2962, 2873, 1664, 1606, 1587, 1504, 1432,1381, 1346, 1246, 1203, 1148, 1090, 753, 591	3430, 3370, 2927, 1650, 1587, 1550, 1450, 1380, 1210, 1162, 752, 523

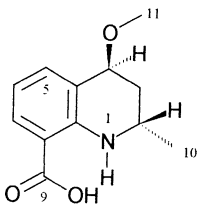
[a]CH_2Cl_2/2 % MeOH, [b]CH_2Cl_2/7 % MeOH

Fig. 3. Structure of helquinoline (**1**) and selected NOESY cross peaks.



1

with the structurally related tetrahydroquinoline L-689,560 (**2**), whose absolute stereochemistry was confirmed by X-ray studies and which exhibited very similar coupling constants. Helquinoline (**1**), however, neither gave a CD effect (in CH_3OH), nor showed it an optical rotation (in $CHCl_3$), pointing to a racemic mixture. HPLC on a chiralcel OD-R column afforded two signals indeed, confirming the presence of a racemate.



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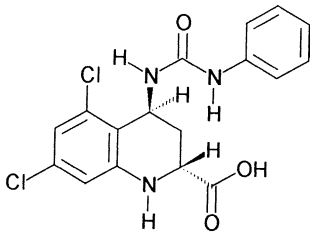

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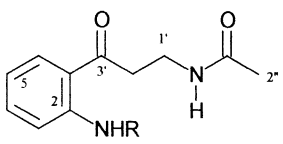
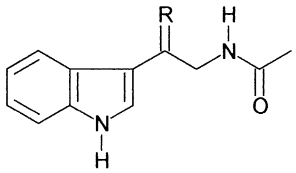
Purification of fraction 3 by HPLC gave compound **3a** along with *p*-hydroxyphenyl-ethanol and N_β-acetyltryptamine (**4a**). Compound **3a** was obtained as a light yellow coloured oil. The EI-MS showed a molecular ion peak at *m/z* 206.3, which was confirmed by ESI-MS with peaks at *m/z* 207.3 $(M+H)^+$, 229.4 $(M+Na)^+$, and 435.0 $(2M+Na)^+$. The HREIMS data of **3a** led to the molecular formula $C_{11}H_{14}O_2N_2$. The ^1H NMR spectrum in deuteriochloroform showed a triplet and a quartet at δ 3.18 and 3.62, respectively. The latter gave a triplet after D_2O exchange, which is typical for an ethanediyl group in vicinal position to NH. The four 1H signals at δ 6.62 (td), 6.64 (dd), 7.26 (td), and 7.71 (dd) suggested the presence of two substituents in *ortho* position to each other and one of them being a strong electron donor. The 3H singlet at δ 1.96 indicated the presence of a methyl group adjacent to a carbonyl group. The ^{13}C NMR spectrum displayed the carbonyl signals of a ketone at δ 201.2 and another one at δ 170.1 due to a carboxylic acid, an ester or amide, six sp^2 carbon signals, one aliphatic CH_3, and two CH_2 signals. Due to the chemical shifts and the formula, the methyl group must be present as an acetyl residue. These fragments can be assembled only in a few different manners, among which N_β-acetylkynuramine (**3a**) is the most plausible one: Oxidative cleavage of N_β-acetyltryptamine (**4a**), which was also isolated here, would deliver N_β-acetyl-*N*-formylkynuramine (**3b**), and this should be easily deformylated into **3a**. Most other combinations of the fragments could not explain the keto carbonyl, would show an aldehyde signal or are not in accordance with the proton shifts. The isolated product has been unambiguously identified as **3a** by comparison with a synthetic sample which was obtained by treatment of **4a** with *m*-chloroperbenzoic acid as described for the degradation of melatonin[20], and by acidic hydrolysis of the resulting **3b**. Several derivatives of kynuramine including **3b**[21] have been described in the literature, however, **3a** has not been mentioned so far and is therefore a new natural product. It has been isolated recently also from an arctic ice bacterium isolate ANT V/2 253 in our group[22].



3a: R = H

3b: R = CHO



4a: R = H_2

4b: R = O

Table 2. Antimicrobial activities of **1** and 8-hydroxyquinolinium sulphate (HQ) in the agar diffusion test (conc. 30 μg per disk, diameter of inhibition zone in mm).

	SA[a]	EC[b]	SV[c]	BS[d]	CA[e]	MM[f]
Crude extract	31	12	25	11	12	30
1	15	0	19	12	0	0
HQ	26	28	26	24	0	30

[a] *Streptococcus aureus*, [b] *Escherichia coli*, [c] *Streptomyces viridochromogenes* Tü57, [d] *Bacillus subtilis*, [e] *Candida albicans*, [f] *Mucor miehei*.

Biological Properties

Antibacterial antifungal and antimicroalgal activities were qualitatively determined using the agar diffusion method with paper disks (i.d. 9 mm). The crude extract showed a higher activity against the test strains than the pure metabolites. Among **1**, **3b** and **4b**, only the first compound **1** showed moderate activity against *Bacillus subtilis* (BS), *Streptomyces viridochromogenes* Tü 57 (SV) and *Staphylococcus aureus* (SA), however was inactive against *Escherichia coli* (EC), *Candida albicans* (CA) and *Mucor miehei* (MM) (Table 2). All three compounds were inactive against the micro-algae *Chlorella vulgaris*, *Chlorella sorokiniana*, and *Scenedesmus subspicatus*, while the crude extract was highly active. As mixing of the isolated metabolites did not enhance their activity, synergistic effects can be excluded. We are searching now for a further labile compound which probably was decomposed during separation.

Experimental

IR-spectra were recorded on a Perkin-Elmer 1600 series FT-IR spectrometer. UV spectra were recorded on a HP8451 A diode array spectrophotometer. ESI-MS was recorded on a Quattro Triple Quadrupole Mass Spectrometer, Finnigen TSQ 7000 with nano-ESI-API-ion source. EI-MS was recorded on a Varian MAT 731 (70 eV) & Varian 311A (70 eV). Perfluorkerosene was used as reference substance in EI-HRMS. NMR spectra were measured on a Varian Unity 300 (300.145 MHz), AMX 300 (300.135 MHz) and a Varian Inova 500 (499.876 MHz) spectrometer. HPLC was performed on a 200×4 mm chiralcel OD-R column (Daicel Chemical Industries, Ltd.). Preparative HPLC was performed using an RP 18 column (Eurochrom Eurospher RP 100-C18, 5 μm) using a Jasco Variable Wavelength Monitor. Flash chromatography was carried out on silica gel (230~400 mesh). Thin layer chromatography (PTC) were performed on Polygram SIL G/UV_{254} (Macherey-Nagel & Co.), R_f values were determined with CH_2Cl_2/7% MeOH. Sephadex LH 20 (Pharmacia) was used for gel filtration.

Fermentation of *Janibacter limosus* strain Hel 1

Well-grown marine broth agar cultures of *Janibacter limosus* Hel 1 served to inoculate 100 ml marine broth medium (250 ml Erlenmeyer flask). After three days incubation at 27 °C and 100 rpm the broth (4×25 ml) was transferred to four 2-liter Erlenmeyer flasks each containing 500 ml of artificial seawater/Luria Bertani medium (10 g/liter tryptone, 5 g/liter yeast extract, 5 g/liter NaCl) supplemented with 50 g/liter glucose. This preculture was kept under the above conditions for 24 hours and was used to inseed a 50-liter bioreactor filled with 38 liters of preculture medium components. The cultivation conditions were as follows: aeration rate 0.3 v/vm, agitation speed 200 rpm (Rushton turbine), 27 °C, initial pH 7.2. After 48 hours the fermentation (no pH correction) was stopped by adjusting the pH to 3.5 (HCl) and centrifugation at 16,000 rpm. The cell pellet was extracted with $CHCl_3$/CH_3OH (2/1, v/v), the supernatant was treated by counter-current extraction with ethyl acetate yielding together 4.62 g of crude extract which was defatted with cyclohexane.

The crude extract (3.55 g) was subjected to silica gel column chromatography (65×3 cm) using a CH_2Cl_2/CH_3OH gradient (7 steps, 1~20% MeOH, each 500 ml) as eluent to give seven fractions four of them were active. The active fraction 1 was purified on Sephadex LH 20 (80×3 cm, MeOH) and PTLC to yield helquinoline (**1**, 4.7 mg) and anthranilic acid (215 mg, R_f=0.71). The fraction 2 on purification by HPLC RP 18 (MeCN/H_2O) delivered cyclo(isoleucylprolyl) (25 mg, R_f=0.66), cyclo(tyrosylprolyl (22 mg, R_f=0.36), and indole-3-carboxyaldehyde (12 mg, R_f=0.73) while fraction 3 on purification by Sephadex LH 20 (CH_2Cl_2/CH_3OH, 3 : 2) followed by HPLC gave *N*-acetylkynuramine (**3a**, 7.5 mg) along with *p*-hydroxyphenylethanol (8.2 mg, R_f=0.47), and N_β-acetyltryptamine (**4a**, 12 mg, R_f=0.49). Fraction 4 on purification by HPLC resulted in the isolation of *N*-acetyl-α-oxotryptamine (**4b**, 12 mg, R_f=0.37) and *p*-hydroxyphenylpropanic acid (14 mg, R_f=0.39). Phenylacetamide and crotonic acid were obtained as a mixture from fraction 5. The fractions 6 and 7 yielded the frequently occurring derivatives indole-3-carboxylic acid (5.3 mg, R_f=0.28) and thymine (11 mg, R_f=0.19).

Helquinoline (**1**)

^{1}H NMR ($CDCl_3$, 300 MHz): δ=7.92 (dd, J=8.1, 1.8 Hz, 1H, 7-H), 7.22 (dd, J=8.1, 1.8 Hz, 1H, 5-H), 6.54 (t, J=8.1 Hz, 1H, 6-H), 4.20 (t, J=2.9 Hz, 1H, 4-H), 3.74 (m, 1H, 2-H), 3.38 (s, 3H, 11-OCH_3), 2.14 (dt, J=13.5, 3.0 Hz, 1H, 3-H_α), 1.48 (td, J=13.5, 2.7 Hz, 1H, 3-H_β), 1.34 (d, J=6.6 Hz, 3H, 10-CH_3). – ^{13}C NMR ($CDCl_3$, 125.7 MHz; assignments from HMQC): δ=173.5 (9-C_q), 148.6 (8a-C_q), 136.9 (5-CH), 133.0 (7-CH), 119.8 (8-C_q), 113.3 (6-CH), 107.9 (4a-C_q), 74.9 (4-CH), 55.3 (11-OCH_3), 41.6 (2-CH), 33.9 (3-CH_2), 22.1 (10-CH_3). – HMBC ($CDCl_3$, IN4LPLRND, F1 125.7 MHz, F2 499.9 MHz) (H→C):

3-H_{α}→C-4; 3-H_{β}→C-2, C-10; 4-H→C-2, C-8a, C-11; 5-H→C-7, C-4, C-8a; 6-H→C-5, C-7, C-8, C-4a; 7-H→C-5, C-9, C-8a; 10-H_3→C-8a, C-2; 11-H_3→C-4. –EI-MS: m/z=221.1 (M^+, 10), 207.1 (16), 188.1 (32), 174.1 (100), 156.1 (48), 144.1 (14), 128.1 (10), 84 (26), 66 (38).

N-Acetylkynuramine (**3a**)

^{1}H NMR ($CDCl_3$, 300 MHz): δ=7.71 (dd, J=8.5, 1.4 Hz, 1H, 3-H), 7.26 (td, J=8.3, 1.5 Hz, 1H, 5-H), 6.64 (dd, J=8.5, J'=1.4 Hz, 1H, 6-H), 6.62 (td, J=8.5, J'=1.5 Hz, 1H, 4-H), 3.62 (q, J=8.0 Hz, 2H, 1′-H_2), 3.18 (t, J=8.0 Hz, 2H, 2′-H_2), 1.96 (s, 3H, 2″-CH_3). –^{1}H NMR (C_6D_6, 500 MHz): δ=7.31 (dd, J=8.2, J'=1.5 Hz, 1H, 3-H), 6.97 (td, J=8.2, J'=1.5 Hz, 1H, 4-H), 6.41 (td, J=8.2, J'=1.4 Hz, 1H, 5-H), 6.14 (dd, J=8.2, J'=1.4 Hz, 1H, 6-H), 5.94 (s br, H/D exchangeable, 2H, NH_2), 5.35 (s br, H/D exchangeable, 1H, NH), 3.45 (dt, J=6.1, J'=6.1 Hz, 2H, 2′-H_2), 2.83 (t, J=6.1 Hz, 2H, 1′-H_2), 1.42 (s, 3H, 2″-CH_3). –^{13}C NMR ($CDCl_3$, 75.5 MHz): δ=201.2 (3′-C_q), 170.1 (1″-C_q), 150.3 (2-C_q), 134.6 (4-CH), 130.9 (6-CH), 117.4 (1-C_q), 117.3 (5-CH), 115.8 (3-CH), 38.4 (1′-CH_2), 34.5 (2′-CH_2), 23.2 (2″-CH_3). –EI-MS: m/z=206.3 (M^+, 10), 207.1 (16), 188.1 (32), 174.1 (100), 156.1 (48), 144.1 (14), 128.1 (10), 84 (26), 66 (38).

Synthesis of **3a** by hydrolysis of **3b**

A solution of 240 mg N_{β}-acetyl-N^2-formylkynuramine (**3b**) in 25 ml EtOH and 0.5 ml 2 N HCl was refluxed for 10 minutes and then evaporated to dryness. Repeated preparative TLC (2 plates 20×20 cm, $CHCl_3$/10% MeOH) delivered 120 mg (56.6%) **3a** as a yellowish oil with the data mentioned above.

Acknowledgments

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