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Quinocidin, a Cytotoxic Antibiotic with an Unusual 3,4-Dihydroquinolizinium Ring and Michael Acceptor Reactivity Toward Thiols

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Abstract: Cytotoxicity-guided fractionation of the culture broth of *Actinomadura* sp. TP-A0019 led to the isolation of quinocidin (1), a cytotoxic antibiotic with an unusual 3,4-dihydroquinolizinium ring. The structural assignment was made on the basis of high-field NMR experiments and chemical synthesis. Comparison of the spectral properties of 1 with those of its synthetic counterparts revealed that 1 is a racemic mixture of two enantiomers, which showed similar cytotoxicity against HeLa-S3 cells. Nucleophile-trapping experiments demonstrated that 1 captured 2-mercaptoethanol and *N*-acetyl-L-cysteine via a Michael addition-type reaction, but was inert toward 2-aminoethanol and glycolic acid. Notably, the addition of 1 to thiols proceeded smoothly in neutral aqueous media at room temperature. In view of the thiol-trapping ability and the unusual structure, 1 provides a unique scaffold for designing drug leads and protein-labeling probes.

Heterocyclic compounds of natural origin play a highly significant role in the drug discovery and development process.^[1] Besides their successful use as drug leads, the intrinsic versatility and physicochemical properties of their heterocyclic rings have attracted growing interest in drug development research.^[2] A recent substructure search using the Drug Data Report database showed that more than 70% of previously approved drugs and over 80% of compounds currently in preclinical trials contain at least one heterocyclic ring.^[1a] Hence, biologically active natural products containing unexplored heterocyclic ring systems provide an opportunity to create a new platform for drug discovery and development. Herein, we report the isolation, structure elucidation, and synthesis of quinocidin (1), a novel cytotoxic antibiotic with a 3,4-dihydroquinolizinium ring, which has hardly been observed in natural products. The unusual heterocyclic system of 1 was demonstrated to capture thiols in neutral aqueous media at room temperature, providing a novel scaffold for the design of drug leads and protein-labeling probes that form covalent bonds with the cysteine residues of their target proteins.^[3]

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Supporting information for this article is given via a link at the end of the document.

Quinocidin (1) was isolated by cytotoxicity-guided fractionation of the culture broth of Actinomadura sp. TP-A0019. Two-step fractionation of the culture broth (300 mL) by flash column chromatography on Diaion HP-20 and ODS followed by reversed-phase HPLC purification afforded 6.6 mg of 1 as a trifluoroacetate salt. The HR-ESI-MS showed a positive molecular ion peak at m/z 270.2219 [M]⁺, consistent with a molecular formula of C₁₉H₂₈N. The ¹H- and ¹³C-NMR spectra of 1 in CD₃OD displayed two sets of signals in a ratio of about 1:1, suggesting the presence of diastereomers or conformers (Table 1). The ¹H-NMR spectrum revealed the presence of five methyl groups (H₃10, H₃16, H₃17, H₃18, and H₃19). The ¹H-¹H COSY spectrum indicated a continued spin system from one of the methyl protons (H₃16) to an olefinic proton (H12) through three methylene protons (H₂13, H₂14, H₂15). Another methyl proton (H_310) showed a COSY correlation to a methine proton (H3), which was further correlated to the methylene protons (H4a and H4b) and the mutually coupled olefinic protons (H1, H2). The coupling constant of the olefinic protons (J = 9.6 Hz for both stereoisomers) suggested a *cis* configuration of the double bond at C1–C2. The other three methyl protons appeared as singlets $(H_317, H_318, and H_319)$ and were assigned by HMBC experiment, which showed correlations from the H₃17 protons to the aromatic C6 carbon and the olefinic C11 and C12 carbons. Moreover, correlations from the H₃18 protons to the C6, C7, and C8 carbons, and from the remaining H₃19 protons to the C8, C9, and C9a carbons allowed the assignment of all aromatic carbons. The HMBC cross-peaks from the H1 proton to the C9a carbon and from the H4a and H4b protons to the C6 carbon, together with the presence of a nitrogen atom in the molecular formula, suggested the presence of a 3,4-dihydroquinolizinium ring in the structure of 1. The E geometry of the double bond at C11-C12 was determined by the strong NOESY correlation between the H₃13 and H₂17 protons. Thus, the planar structure of 1 was established as shown in Figure 1 (right). Such 3,4dihydroquinolizinium scaffold has very rarely been observed in natural products, while quinolizinium and 1,2,3,4-tetrahydroquionolizinium are present in a variety of alkaloids.[4]



Figure 1. Left: structure of quinocidin (1). Right: key 2D NMR correlations.

	Rotamer A		Rotamer B	
position	$\delta_{\rm H}$ (m, J in Hz)	δ_{C}	δ_{H} (m, J in Hz)	$\delta_{\rm C}$
1	6.99 (d, 9.7)	120.6/	6.99 (d, 9.7)	120.6/
		120.5 ^[b]		120.5 ^[b]
2	6.85 (dd, 9.7, 4.4)	145.9	6.87 (dd, 9.7, 4.5)	145.9
3	2.98 (br. m)	30.3	2.98 (br. m)	30.0
4a	4.42 (dd, 13.8, 8.4)	57 5	4.35 (dd, 13.9, 8.0)	57 A
4b	4.51 (dd, 13.8, 5.5)	57.5	4.59 (dd, 13.9, 5.5)	57.4
6		157.2		157.2
7		137.2		137.2
8	8.22/8.23 ^[a] (s)	150.0/	8.22/8.23 ^[a] (s)	150.0/
		149.9 ^[c]		149.9 ^[c]
9		135.2		135.2
9a		145.8		145.8
10	1.17 (d, 7.2)	16.7	1.18 (d, 7.2)	16.8
11		129.1		128.9
12	5.76 (tq, 7.4, 1.3)	140.3/	5.80 (tq, 7.2, 1.3)	140.3/
		140.2 ^[a]		140.2 ^[0]
13	2.41 (m)	29.6	2.41 (m)	29.6
14	1.55 (m)	32.8	1.55 (m)	32.7
15	1.47 (m)	24.5	1.47 (m)	24.5
16	1.01 (t, 7.3)	15.1	1.01 (t, 7.3)	15.1
17	2.04 (s)	16.5	2.03 (s)	17.1
18	2.41 (s)	20.0/	2.41 (s)	20.0/
		19.9 ^[e]		19.9 ^[e]
19	2.54 (s)	19.0	2.54 (s)	19.0
al, [0], [0], [0], [0], [0], [0], [0], [0]				

Table 1. ¹H- and ¹³C-NMR (CD₃OD, 600 and 150 MHz) assignments for two rotamers of quinocidin (1).

and be assigned.

Careful analysis of the NOESY spectrum suggests that the two isomers differed by the orientation of the alkenyl side chain (rotamers A and B; Figure 2). In rotamer A, the H4b proton showed strong NOESY correlations with the H3 and H₃17 protons. On the other hand, the H4b proton of rotamer B showed NOESY correlation with the H3 proton, but not with the H₃17 protons; moreover, a strong correlation between the H4a and H₃17 protons was observed. These results confirm that doubling of the NMR signals was due to the presence of two rotamers, resulting from hindered rotation around the C6–C11 bond. Signal coalescence did not occur even at 60 °C, indicating a considerably high rotation barrier.

In order to confirm the structure of **1** and determine the C3 stereochemistry, the 3S and 3*R* isomers of **1** were synthesized separately. The synthesis of the 3S isomer started with the preparation of alkyne **4**, shown in Scheme 1. After protection of the hydroxyl group of commercially available methyl (*R*)-hydroxyisobutyrate (**2**) with a TBDPS (*tert*-butyldiphenylsilyl) group, the ester was reduced with DIBAL-H (diisobutylaluminium hydride). The resulting alcohol was oxidized to aldehyde **3** under Parikh-Doering conditions^[5] (78% over three steps), and the following Corey-Fuchs one-carbon homologation^[6] provided the desired alkyne **4** (66%), which was used for Sonogashira coupling.^[7] The other coupling partner, i.e., 2-chloropyridine



Figure 2. Key NOESY correlations in rotamers A and B of 1. The 3*R* isomer is shown as an example.



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Scheme 1. Synthesis of alkyne **4**. Reagents and conditions: (a) (i) TBDPS-CI, imidazole, DMF, rt; (ii) DIBAL-H, toluene, -78 °C to rt; (iii) SO₃ pyridine, Et₃N, DMSO, CH₂Cl₂, 0 °C to rt (78%). (b) (i) PPh₃, CBr₄, CH₂Cl₂, 0 °C; (ii) *n*-BuLi, THF, -78 °C to rt (66%).

derivative 8 was prepared from 3,5-lutidine (5) (Scheme 2). Chlorination via regioselective lithiation at the C2 position of 5 using the superbase n-BuLi-Me₂N(CH₂)₂OLi gave the 2-chloro derivative 6 (92%).^[8] Lithiation at the C6 position^[9] of 6 followed by reaction with 2-heptanone provided tertiary alcohol 7 (35%). Next, dehydration with H₂SO₄ and AcOH produced a ~10:3 mixture of the desired alkene 8 and its regioisomer 9, and subsequent isomerization with n-BuLi afforded 8 in 49% yield over two steps. Sonogashira coupling between 2-chloropyridine derivative 8 and alkyne 4 followed by desilylation gave 10 (55% over two steps), which was reduced to the corresponding cisalkene **11** by hydrogenation over Lindlar catalyst (67%). Finally, cyclization was achieved via the mesylate intermediate to provide the desired 3S isomer of 1 (45%) as a trifluoroacetate salt. The 3R isomer was similarly prepared using methyl (S)hydroxyisobutyrate as a starting material (Supporting Information).



Scheme 2. Synthesis of the 3S isomer of 1. Reagents and conditions: (a) *n*-BuLi-Me₂N(CH₂)₂OLi, *n*-hexane, 0 °C then C₂Cl₆, -78 °C (92%). (b) *n*-BuLi-Me₂N(CH₂)₂OLi, *n*-hexane, 0 °C then 2-heptanone, -30 °C to rt (35%). (c) H₂SO₄, AcOH, 70 °C. (d) *n*-BuLi, *n*-hexane, 0 °C (49% over two steps). (e) (i) **4**, PdCl₂(PPh₃)₂, PPh₃, Et₂NH, DMF, 120 °C; (ii) TBAF, THF, rt (55%). (f) H₂, Lindlar catalyst, quinoline, MeOH, rt (67%). (g) MsCl, Et₃N, CH₂Cl₂, -20 °C (45%).

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Figure 3. Structures of 12 and 13.

The ¹H- and ¹³C-NMR spectra of the synthetic 3S and 3R isomers were in perfect agreement with those of isolated 1. However, the specific rotation of **1** ($[\alpha]_D^{25} = -1.9$, c = 0.35, MeOH) was found to be inconsistent with that of either of the two isomers ($[\alpha]_{D}^{25}$ = +42.5, *c* = 0.33, MeOH for the 3S isomer; $[\alpha]_{D}^{25}$ = -45.4, c = 0.26, MeOH for the 3R isomer). The low specific rotation of the natural sample indicates that 1 is a racemic mixture, confirming the structure of 1 as shown in Figure 1 (left). The racemate formation seems to be, at least in part, abiotic since two-month storage of the 3S isomer at -20 °C led to a decreased specific rotation ($[\alpha]_D^{25}$ = +24.2, *c* = 0.09, MeOH). On the other hand, to investigate the effect of the alkenyl side chain on the conformation of 1, the corresponding compound lacking the alkenyl side chain (12) was prepared (Figure 3). As expected, only one set of signals was detected in the ¹H- and ¹³C-NMR spectra of 12 (Supporting Information), supporting our conclusion that the duplicate signals of ${\bf 1}$ derived from the orientation of the alkenyl side chain.

Next, **1** and its synthetic isomers were evaluated for their cytotoxicity against HeLa-S3 cells derived from human cervical carcinoma. Their activities were almost indistinguishable ($IC_{50} = 0.63 \ \mu g/mL$ for **1**, 0.60 $\mu g/mL$ for the 3*S* isomer, and 0.64 $\mu g/mL$ for the 3*R* isomer), clearly indicating the trivial role of the C3 stereochemistry on the cytotoxic activity of **1**. On the other hand, **12**, which lacks the alkenyl side chain, and **13**, which contains a pyridine ring instead of the 3,4-dihydroquinolizinium ring (Figure 3; for its preparation, see Supporting Information), exhibited significantly lower activities ($IC_{50} = >30 \ \mu g/mL$). These combined results suggest that the 3,4-dihydroquinolizinium ring is necessary but insufficient for the cytotoxic activity of **1**.



Figure 4. HPLC chromatograms of the reaction mixtures of (A) 2-aminoethanol, (B) glycolic acid, (C) 2-mercaptoethanol with the synthetic 3R isomer of 1.



Figure 5. HPLC chromatograms of the reaction mixtures of 2mercaptoethanol with 15.

It is well known that α,β -unsaturated iminium salts are highly nucleophilic attack.[10] susceptible to The 3.4dihydroquinolizinium ring includes the α,β -unsaturated iminium moiety (C2-C1-C9a-N5), raising the possibility of adduct formation between 1 and nucleophiles. To explore this aspect, the reactivity of 1 toward 2-aminoethanol, glycolic acid, and 2mercaptoethanol was examined. After treatment of the 3R isomer of 1 with 10 equivalents of each nucleophile in PBS (phosphate-buffered saline, pH 7.4) at room temperature for 2, 4, 8, 24, and 48 h, the reaction mixture was analyzed by HPLC (Figure 4). Intriguingly, whereas the 3R isomer of 1 was inert to 2-aminoethanol and glycolic acid, time-dependent adduct formation was clearly observed in the reaction with 2mercaptoethanol. In a scale-up experiment, the adduct could be identified as 14 (for structural determination, see Supporting Information), suggesting that the reaction proceeded via a Michael addition-type mechanism. Notably, under the same conditions, N-acetyl-L-cysteine also afforded the corresponding adduct (Supporting Information). To our knowledge, this is the first demonstration that the 3,4-dihydroquinolizinium ring can capture thiols in neutral aqueous media at room temperature. Moreover, rapid adduct formation was observed in the reaction of 2-mercaptoethanol with the core structure 15 (Figure 5; for details, see Supporting Information), suggesting the possibility that the reactivity might be controlled by the steric hindrance at the C3 position.

In conclusion, quinocidin (1), a novel 3,4-dihydroquinolizinium compound with cytotoxicity against HeLa-S3 tumor cells, was isolated from the culture broth of Actinomadura sp. TP-A0019. This simple but unusual heterocyclic antibiotic can be easily synthesized and thus can be tuned for optimal pharmacological properties, providing a new platform for the development of anticancer drugs. Of particular interest is the ability of the 3,4dihydroquinolizinium ring to capture thiols in neutral aqueous solution. Although a variety of electrophiles have been used as protein-labeling probes,^[3] warheads in the 3.4dihydroquinolizinium ring has not been observed to date in the system. In particular, the presence of the positive charge is an intriguing feature of this electrophile, suggesting its potential as a novel warhead with a preference for cysteine residues near negatively charged amino acid residues. Further studies of the biosynthesis, the correlation between thiol-trapping ability and cytotoxicity, and the electrophilic reactivity of 1 are in progress.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: Drug discovery • Nitrogen heterocycles • Michael addition • Natural products • Structure elucidation

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