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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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 $C_{19}H_{28}N$. The ^{1}H- and ^{13}C-NMR spectra of **1** in CD_3OD displayed two sets of signals in a ratio of about 1:1, suggesting the presence of diastereomers or conformers (Table 1). The ^{1}H-NMR spectrum revealed the presence of five methyl groups ($H_3$10, $H_3$16, $H_3$17, $H_3$18, and $H_3$19). The ^{1}H-^{1}H COSY spectrum indicated a continued spin system from one of the methyl protons ($H_3$16) to an olefinic proton (H12) through three methylene protons ($H_2$13, $H_2$14, $H_2$15). Another methyl proton ($H_3$10) showed a COSY correlation to a methine proton (H3), which was further correlated to the methylene protons (H4a and H4b) and the mutually coupled olefinic protons (H1, H2). The coupling constant of the olefinic protons (J = 9.6 Hz for both stereoisomers) suggested a *cis* configuration of the double bond at C1–C2. The other three methyl protons appeared as singlets ($H_3$17, $H_3$18, and $H_3$19) and were assigned by HMBC experiment, which showed correlations from the $H_3$17 protons to the aromatic C6 carbon and the olefinic C11 and C12 carbons. Moreover, correlations from the $H_3$18 protons to the C6, C7, and C8 carbons, and from the remaining $H_3$19 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 $H_3$13 and $H_2$17 protons. Thus, the planar structure of **1** was established as shown in Figure 1 (right). Such 3,4-dihydroquinolizinium 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]

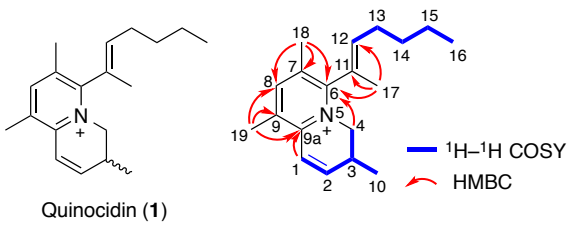


Figure 1. Left: structure of quinocidin (**1**). Right: key 2D NMR correlations.

Table 1. ^{1}H- and ^{13}C-NMR (CD_3OD, 600 and 150 MHz) assignments for two rotamers of quinocidin (**1**).

	Rotamer A		Rotamer B	
position	δ_H (m, *J* in Hz)	δ_C	δ_H (m, *J* in Hz)	δ_C
1	6.99 (d, 9.7)	120.6/ 120.5[b]	6.99 (d, 9.7)	120.6/ 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)	57.4
4b	4.51 (dd, 13.8, 5.5)		4.59 (dd, 13.9, 5.5)	
6		157.2		157.2
7		137.2		137.2
8	8.22/8.23[a] (s)	150.0/ 149.9[c]	8.22/8.23[a] (s)	150.0/ 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/ 140.2[d]	5.80 (tq, 7.2, 1.3)	140.3/ 140.2[d]
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/ 19.9[e]	2.41 (s)	20.0/ 19.9[e]
19	2.54 (s)	19.0	2.54 (s)	19.0

[a],[b],[c],[d],[e]The signals of rotamers A and B could not 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 $H_3$17 protons. On the other hand, the H4b proton of rotamer B showed NOESY correlation with the H3 proton, but not with the $H_3$17 protons; moreover, a strong correlation between the H4a and $H_3$17 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 3*S* and 3*R* isomers of **1** were synthesized separately. The synthesis of the 3*S* 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

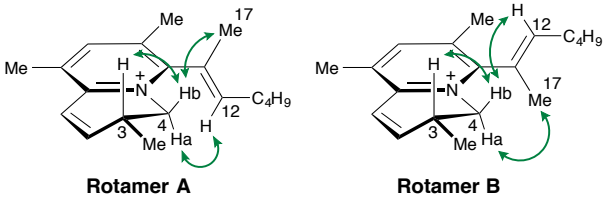
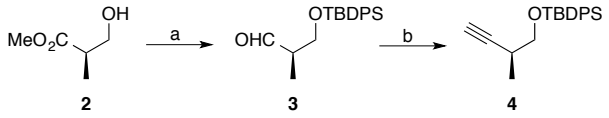
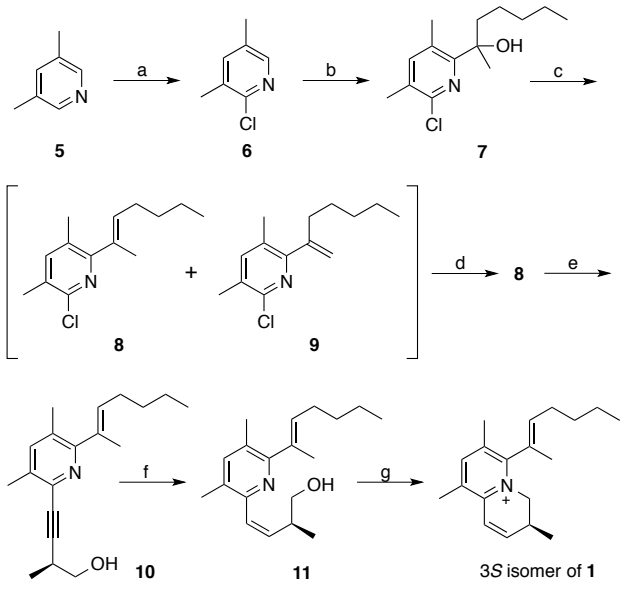


Figure 2. Key NOESY correlations in rotamers A and B of **1**. The 3*R* isomer is shown as an example.

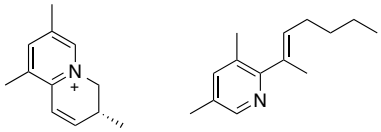


Scheme 1. Synthesis of alkyne **4**. Reagents and conditions: (a) (i) TBDPS-Cl, imidazole, DMF, rt; (ii) DIBAL-H, toluene, –78 °C to rt; (iii) SO_3·pyridine, Et_3N, DMSO, CH_2Cl_2, 0 °C to rt (78%). (b) (i) PPh_3, CBr_4, CH_2Cl_2, 0 °C; (ii) *n*-BuLi, THF, –78 °C to rt (66%).

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–$Me_2N(CH_2)_2OLi$ 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 H_2SO_4 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 *cis*-alkene **11** by hydrogenation over Lindlar catalyst (67%). Finally, cyclization was achieved via the mesylate intermediate to provide the desired 3*S* isomer of **1** (45%) as a trifluoroacetate salt. The 3*R* isomer was similarly prepared using methyl (*S*)-hydroxyisobutyrate as a starting material (Supporting Information).



Scheme 2. Synthesis of the 3*S* isomer of **1**. Reagents and conditions: (a) *n*-BuLi–$Me_2N(CH_2)_2OLi$, *n*-hexane, 0 °C then C_2Cl_6, –78 °C (92%). (b) *n*-BuLi–$Me_2N(CH_2)_2OLi$, *n*-hexane, 0 °C then 2-heptanone, –30 °C to rt (35%). (c) H_2SO_4, AcOH, 70 °C. (d) *n*-BuLi, *n*-hexane, 0 °C (49% over two steps). (e) (i) **4**, $PdCl_2(PPh_3)_2$, PPh_3, Et_2NH, DMF, 120 °C; (ii) TBAF, THF, rt (55%). (f) H_2, Lindlar catalyst, quinoline, MeOH, rt (67%). (g) MsCl, Et_3N, CH_2Cl_2, –20 °C (45%).



12 **13**

Figure 3. Structures of **12** and **13**.

The ^{1}H- and ^{13}C-NMR spectra of the synthetic 3*S* and 3*R* 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 3*S* isomer; $[\alpha]_D^{25}$ = –45.4, *c* = 0.26, MeOH for the 3*R* 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 3*S* 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 ^{1}H- and ^{13}C-NMR spectra of **12** (Supporting Information), supporting our conclusion that the duplicate signals of **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 μg/mL for **1**, 0.60 μg/mL for the 3*S* isomer, and 0.64 μg/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 μg/mL). These combined results suggest that the 3,4-dihydroquinolizinium ring is necessary but insufficient for the cytotoxic activity of **1**.

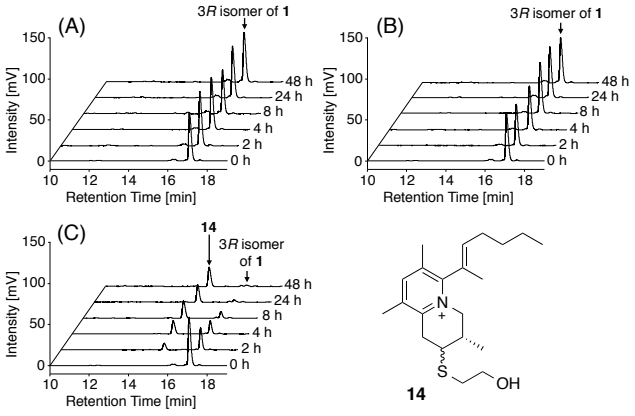


Figure 4. HPLC chromatograms of the reaction mixtures of (A) 2-aminoethanol, (B) glycolic acid, (C) 2-mercaptoethanol with the synthetic 3*R* isomer of **1**.

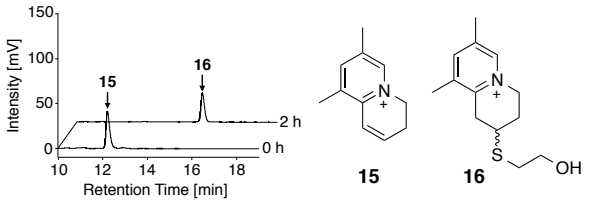


Figure 5. HPLC chromatograms of the reaction mixtures of 2-mercaptoethanol with **15**.

It is well known that α,β-unsaturated iminium salts are highly susceptible to nucleophilic attack.[10] The 3,4-dihydroquinolizinium 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 2-mercaptoethanol was examined. After treatment of the 3*R* 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 3*R* isomer of **1** was inert to 2-aminoethanol and glycolic acid, time-dependent adduct formation was clearly observed in the reaction with 2-mercaptoethanol. 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 3,4-dihydroquinolizinium ring to capture thiols in neutral aqueous solution. Although a variety of electrophiles have been used as warheads in protein-labeling probes,[3] the 3,4-dihydroquinolizinium 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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