# Relationship Among Structure, Cytotoxicity, and Michael Acceptor Reactivity of Quinocidin

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**ABSTRACT**

Quinocidin (QCD) is a cytotoxic antibiotic with an unusual 3,4-dihydroquinolizinium skeleton. We previously found that QCD captures thiols in neutral aqueous media *via* a Michael addition-type reaction. However, it remains unclear whether the Michael acceptor reactivity of QCD is responsible for its cytotoxicity. In this study, we synthesized thirteen analogs of QCD to examine the relationship among its structure, cytotoxicity, and reactivity toward thiols. Thiol-trapping experiments and cytotoxicity tests collectively suggested that the Michael acceptor function of QCD is independent of its cytotoxic activity, and that the pyridinium moiety with the hydrophobic side chain is a key structural factor for cytotoxicity. These findings further led us to demonstrate that incorporation of an amide group into the side chain of QCD significantly reduced its toxicity but hardly affected the Michael acceptor function. The present study lays the foundation for QCD-based drug design and highlights the potential of QCD as a unique electrophile for use in the development of covalent inhibitors and protein-labeling probes.

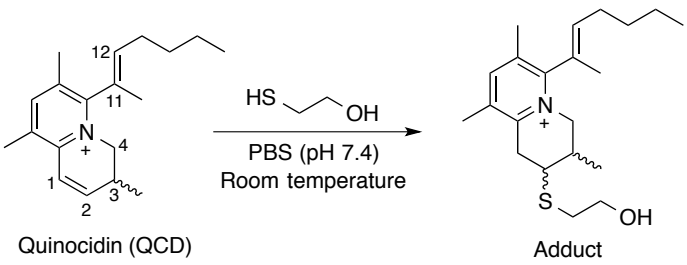
**KEYWORDS**

Antibiotic, Anticancer, Heterocycle, Michael addition, Structure–activity relationship

## 1. Introduction

Heterocyclic rings are well established as fundamental elements in drug design.[1–4] A recent substructure analysis using the Drug Data Report database revealed the increasing use of heterocyclic rings in medicinal chemistry.[1] More than 70% of the previously approved drugs and over 80% of the drug candidates currently in preclinical testing are heterocyclic compounds. It is remarkable that most of them are derived from or inspired by heterocyclic fragments of natural products. Hence, natural products with unexplored heterocyclic rings provide an opportunity to uncover a new repertoire of drug scaffolds.

Recently, we isolated quinocidin (QCD; Scheme 1) from the culture broth of *Actinomadura* sp. TP-A0019 as a cytotoxic compound against HeLa-S3 cells.[5] QCD has a 3,4-dihydroquinolizinium ring, which is very rarely observed in natural products, unlike quinolizinium and 1,2,3,4-tetrahydroquinolizinium rings. Of further significance, the 3,4-dihydroquinolizinium moiety of QCD captures thiols in neutral aqueous media *via* a Michael addition-type reaction (Scheme 1). The unusual heterocyclic ring and unique Michael acceptor function of QCD, together with its cytotoxicity against cancer cells, suggest its great potential as a novel lead or scaffold for designing drug candidates.




**Scheme 1.** Addition reaction of quinocidin (QCD) with 2-mercaptoethanol.

One prerequisite for the future application of QCD in pharmaceutical research is to define the correlation between its Michael acceptor function and cytotoxicity. We have previously shown that a QCD analog (**1**; Fig. 1) containing a pyridine ring instead of the 3,4-dihydroquinolizinium ring exhibited significantly lower toxicity against HeLa-S3 cells ($IC_{50}$ > 30 μM).[5] Although this finding suggests the possible importance of the 3,4-dihydroquinolizinium ring of QCD for cytotoxicity, the contribution of the Michael acceptor function cannot be estimated. In this study, we address this issue by synthesizing thirteen QCD analogs (**2**–**14**; Fig. 1) and evaluating their cytotoxicity against HeLa-S3 cells and reactivity toward 2-mercaptoethanol. We also report the development of a non-toxic QCD analog (**29**) retaining the Michael acceptor function, as a preliminary study toward the future application of QCD in the design of covalent inhibitors and protein-labeling probes.[6–9]

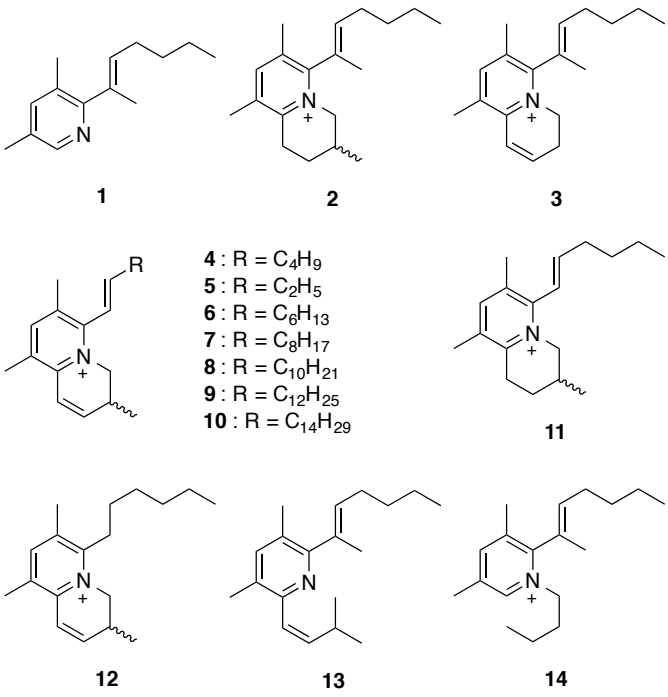
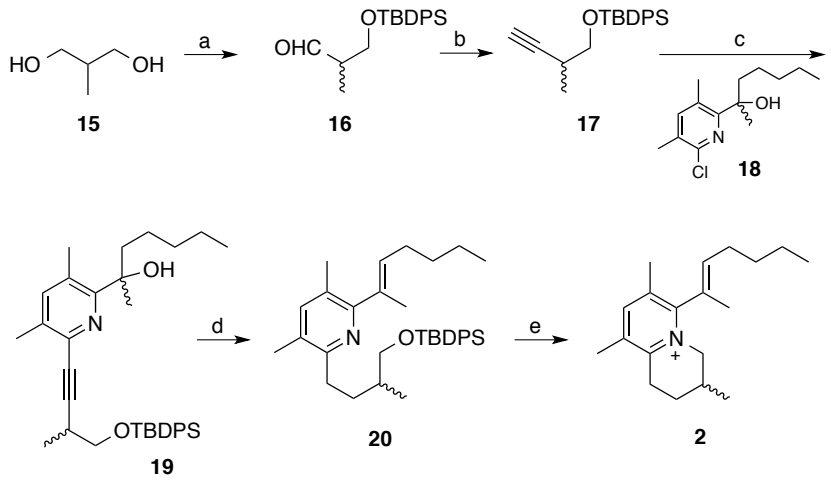



**Fig. 1.** Structures of QCD analogs.

## 2. Results and discussion

### *2.1. Synthesis and evaluation of 1,2-dihydro analog of QCD (2)*

We initially prepared the 1,2-dihydro analog of QCD (**2**), which lacks the thiol-reactive double bond, to examine the relationship between the Michael acceptor function and the cytotoxicity of QCD. The synthesis of **2** was initiated with the monoprotection of 2-methyl-1,3-propanediol **15** with a TBDPS (*tert*-butyldiphenylsilyl) group and oxidation of the remaining hydroxyl group to generate aldehyde **16** (Scheme 2). Corey–Fuchs one-carbon homologation[10] of **16** provided alkyne **17**, which was coupled with the previously prepared chloropyridine unit **18**[5] under typical Sonogashira conditions.[11] Hydrogenation of coupling product **19**, followed by dehydration with the Burgess reagent[12] gave alkene **20**. Finally, desilylation and cyclization *via* the mesylate intermediate afforded **2** as a trifluoroacetate salt.




**Scheme 2.** Synthesis of **2**. Reagents and conditions: a) (i) TBDPS-Cl, NaH, THF, rt; (ii) $SO_3$·pyridine, $Et_3N$, DMSO, $CH_2Cl_2$, 4 °C to rt (89% in 2 steps). b) (i) $PPh_3$, $CBr_4$, $CH_2Cl_2$, 4 °C; (ii) *n*-BuLi, THF, –78 °C to rt (95% in 2 steps). c) **18**, CuI, $PdCl_2(PPh_3)_2$, $Et_2NH$, $PPh_3$, DMF, 120 °C (41%). d) (i) $H_2$, 5% Pd-C, EtOH, rt; (ii) Burgess reagent, toluene, 80 °C (20% in 2 steps). e) (i) TBAF, THF, rt; (ii) MsCl, $Et_3N$, $CH_2Cl_2$, 4 °C to rt (83% in 2 steps).

We then examined whether **2** is unresponsive to thiols. In our previous study, when QCD was treated with 10 equivalents of 2-mercaptoethanol in PBS (phosphate-buffered saline, pH 7.4) at room temperature, time-dependent adduct formation was clearly observed by HPLC analysis (Fig. 2; A).[5] Thus, the reactivity of **2** toward 2-mercaptoethanol was evaluated under the same conditions. HPLC analysis of the reaction mixture showed that **2** remained unchanged, and adduct formation was hardly observed even after 24 h (Fig. 2B), suggesting a lack of the Michael acceptor function of **2**. Having confirmed that **2** can act as a QCD analog without showing any reactivity toward thiols, we subsequently evaluated its toxicity against HeLa-S3 cells. Unexpectedly, cytotoxicity of **2** ($IC_{50}$ = 0.36 μM) was slightly higher than that of QCD ($IC_{50}$ = 0.64 μM). These results indicated that the cytotoxicity of QCD is independent of the Michael acceptor function.

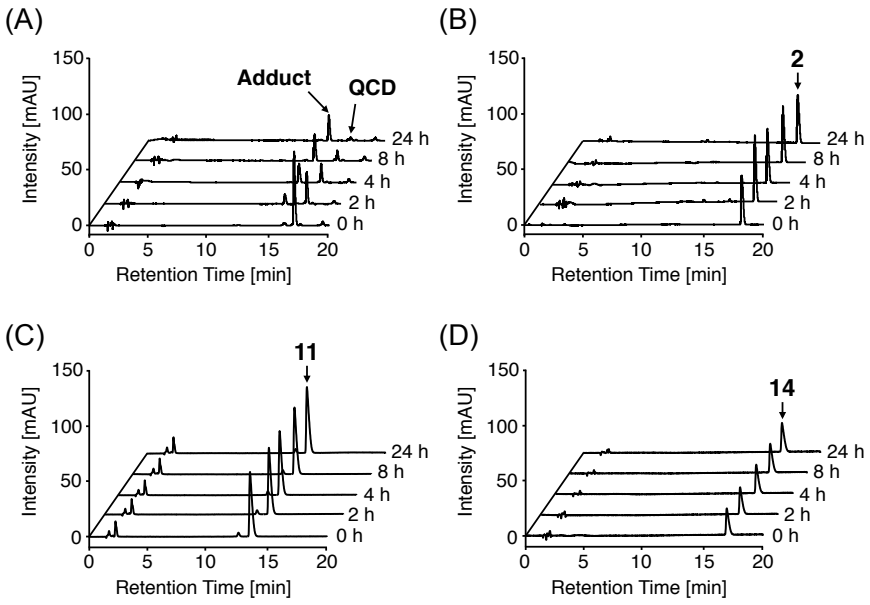
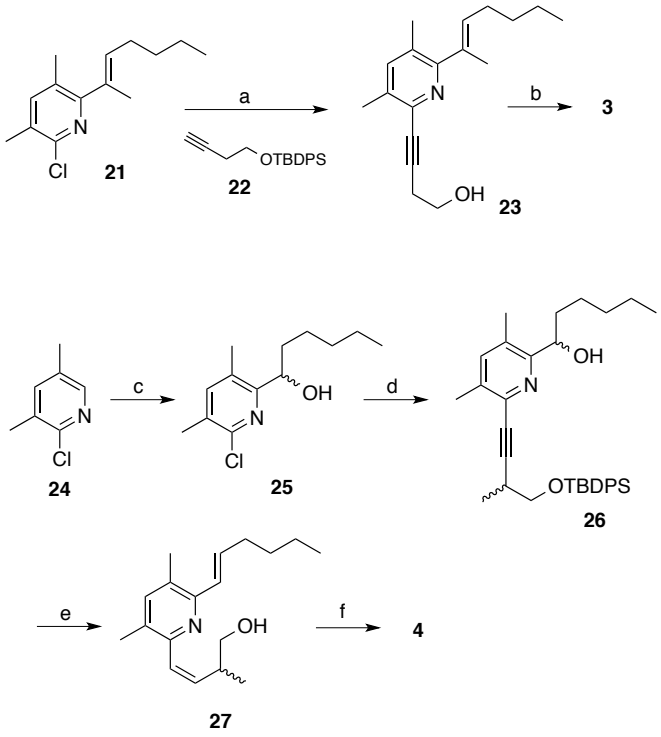



**Fig. 2.** HPLC chromatograms of the reaction mixtures of 2-mercaptoethanol with QCD (A), **2** (B), **11** (C), and **14** (D).

*2.2. Synthesis and evaluation of QCD analogs (**3**–**14**)*

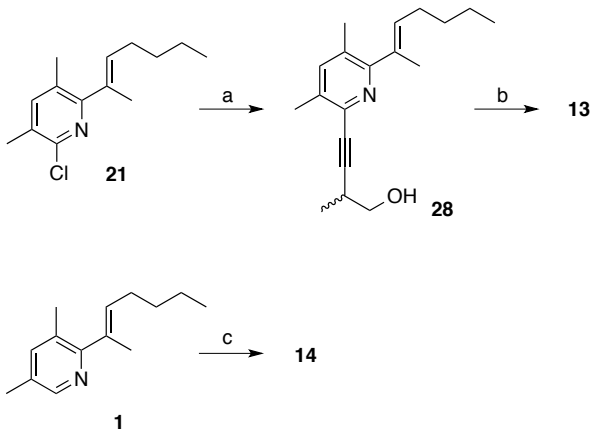
The above finding prompted us to explore the structural requirements for the cytotoxicity of QCD. Thus, we designed an additional twelve QCD analogs (**3**–**14**; Fig. 1) with modification of the 3,4-dihydroquinolizinium moiety and/or the alkenyl side chain. 3-Demethyl analog **3** was obtained from the synthetic intermediate of QCD (**21**)[5] in 4 steps (Scheme 3; upper). Sonogashira coupling with known alkyne **22**,[13] followed by desilylation, gave alkynyl alcohol **23**. After the triple bond of **23** was reduced by hydrogenation over Lindlar catalyst, the 3,4-dihydropyrizinium ring was constructed *via* mesylation to afford **3**.




**Scheme 3.** Synthesis of **3** (upper) and **4** (lower). Reagents and conditions: a) (i) **22**, CuI, $PdCl_2(PPh_3)_2$, $Et_2NH$, $PPh_3$, DMF, 120 °C; (ii) TBAF, THF, rt (49% in 2 steps). b) (i) $H_2$, Lindlar cat., quinoline, MeOH, rt; (ii) MsCl, $Et_3N$, $CH_2Cl_2$, –20 °C (23% in 2 steps). c) *n*-BuLi, $Me_2N(CH_2)_2OH$, *n*-hexane, 0 °C, then *n*-hexanal, –30 °C to rt (40%). d) **17**, CuI, $PdCl_2(PPh_3)_2$, $Et_2NH$, $PPh_3$, DMF, 120 °C (51%). e) (i) $H_2$, Lindlar cat., quinoline, MeOH, rt; (ii) Burgess reagent, toluene, 80 °C; (iii) TBAF, THF, rt (51% in 3 steps). f) MsCl, $Et_3N$, $CH_2Cl_2$, –20 °C (63%)

On the other hand, the synthesis of 11-demethyl analog **4** was initiated with chlorolutidine **24**,[14] which was converted into alcohol **25** in three steps involving lithiation with the *n*-BuLi-$Me_2N(CH_2)_2OLi$ superbase and nucleophilic addition to 1-hexanal (Scheme 3; lower). Sonogashira coupling with **17**, followed by hydrogenation, dehydration, and desilylation, provided cyclization precursor **27**. Finally, mesylate-mediated cyclization was conducted to produce **4**. Based on the synthetic routes to **3** and **4**, QCD analogs with different lengths of alkenyl side chains (**5**–**10**), 1,2-dihydro-11-demethyl analog (**11**), and 11,12-dihydro-11-demethyl analog (**12**) were similarly prepared (for details, see Supplementary material).

The non-cationic analog (**13**; Fig. 1) was also synthesized using building blocks **17** and **21** (Scheme 4; upper). Sonogashira coupling, followed by desilylation, provided alkynyl alcohol **28**. Dehydroxylation of **28** was achieved by a two-step mesylation/reduction procedure. The triple bond of the resulting deoxy derivative was then reduced by semi-hydrogenation over Lindlar catalyst to furnish **13**. On the other hand, pyridinium analog **14** was readily prepared by *N*-butylation of **1** (Scheme 4; lower).




**Scheme 4.** Synthesis of **13** (upper) and **14** (lower). a) (i) **17**, CuI, $PdCl_2(PPh_3)_2$, $Et_2NH$, $PPh_3$, DMF, 120 °C; (ii) TBAF, THF, rt (43% in 2 steps). b) (i) MsCl, $Et_3N$, $CH_2Cl_2$, 4 °C; (ii) $NaBH_4$, DMF, rt; (iii) $H_2$, Lindlar cat., quinoline, MeOH, rt (25% in 3 steps). c) 1-iodobutane, toluene, 140 °C (71%).

**Table 1**

Cytotoxicity of QCD and its analogs (**1**–**14**) against HeLa-S3 cells

| Compound | $IC_{50}$ (μM)[a] |
|---|---|
| QCD | 0.64 |
| **2** | 0.36 |
| **3** | 0.68 |
| **4** | 0.37 |
| **5** | 2.0 |
| **6** | 0.17 |
| **7** | 0.051 |
| **8** | 0.052 |
| **9** | 0.038 |
| **10** | 0.058 |
| **11** | 0.58 |
| **12** | 0.47 |
| **13** | > 30 |
| **14** | 0.72 |

[a]Concentration (μM) required for 50% growth inhibition of HeLa-S3 cells. $IC_{50}$ values were determined from the corresponding dose–response curves (see Supplementary material).

Table 1 summarizes the cytotoxicity ($IC_{50}$ values) of the QCD analogs (**2**–**14**) against HeLa-S3 cells. Demethyl analogs (**3**, **4**) showed a similar level of activity to that of QCD. When compared to **4**, its 1,2-dihydro and 11,12-dihydro analogs (**11**, **12**) exhibited slightly lower but still very similar potency. These results suggest that the 3- and 11-methyl groups and 1,2- and 11,12-double bonds of QCD hardly contribute to its cytotoxicity. The trivial role of the 3-methyl group and 1,2-double bond is also supported by the similar $IC_{50}$ values of QCD and **14**, which lacks the corresponding moieties. On the other hand, non-cationic analog **13** showed significantly lower activities. The sharp difference in potency between ring-opening analogs **13** and **14** suggests that the cytotoxicity of QCD originates from the pyridinium form, but not simply from the pyridine ring. Regarding the side chain-modified analogs (**4**–**10**), there was a clear correlation

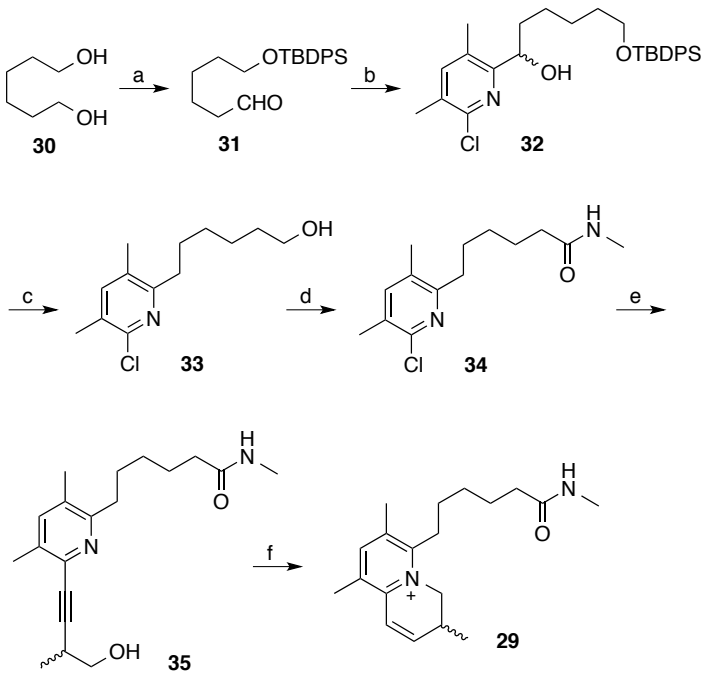
between chain length and cytotoxicity, i.e., the potency increased in the order of the C4 (**5**) < C6 (**4**) < C8 (**6**) < C10 (**7**) alkenyl chains. Further elongation of the side chain from C12 to C16 (**8**, **9**, **10**) had very little effect on the potency. These results collectively indicate that the cytotoxicity of QCD depends on the hydrophobicity of the alkenyl side chain, and that its potency can be enhanced almost 10-fold by the elongation of the alkenyl chain.

*2.3. Design, synthesis, and evaluation of amide-containing QCD analog (**29**)*

The non-dependence between the cytotoxicity and the thiol-trapping ability of QCD was further confirmed by the lack of reactivity of **11** and **14** toward 2-mercaptoethanol (Fig. 2; C, D), although both these compounds exhibited cytotoxicity comparable to that of QCD. This raises the possibility that non-toxic QCD analogs with a Michael acceptor function could be designed and potentially used as electrophiles for covalent inhibitors or protein-labeling probes. As a preliminary study in this regard, we synthesized model analog **29** with an amide-containing side chain (Scheme 5). The molecular design of **29** is based on two assumptions: 1) incorporation of the hydrophilic amide group into the side chain could greatly suppress the cytotoxicity because the cytotoxic activity of QCD depends on hydrophobicity of its side chain, and 2) when considering the connection of protein ligands with QCD, the amide group would be one of the promising linkages.

The synthesis of **29** is shown in Scheme 5. Monoprotection and oxidation of 1,6-hexanediol **30** generated aldehyde **31**,[15] which was then reacted with lithiated **24**. Two-step deoxygenation of the resulting alcohol **32**, followed by desilylation, provided chloropyridine unit **33** bearing a hexanol side chain. The *N*-methyl amide moiety was constructed through oxidation and coupling with methylamine to give **34**. The desired analog **29** was finally obtained through Sonogashira coupling of **34** with **17**, semi-hydrogenation, and mesylate-mediated cyclization. The thiol-

trapping experiment and cytotoxicity test revealed that **29** formed adduct **36** with 2-mercaptoethanol (Fig. 3) but hardly showed toxicity against HeLa-S3 cells ($IC_{50}$ > 30 μM), supporting our design strategy. Additional preliminary experiments demonstrated that **29** also reacted with glutathione containing a cysteine moiety (see, Supplementary material), and was non-toxic against non-cancerous human embryonic kidney (HEK293) cells ($IC_{50}$ > 30 μM) unlike QCD ($IC_{50}$ = 1.7 μM). Although further studies regarding cell permeability and reactivity toward cysteine-containing proteins are still required, these results indicate the potential of QCD as a novel electrophile for covalent protein inhibition or protein-labeling in living cells.




**Scheme 5.** Synthesis of **29**. a) (i) TBDPS-Cl, NaH, DMF, rt; (ii) $SO_3$·pyridine, $Et_3N$, DMSO, $CH_2Cl_2$, 4 °C to rt (59% in 2 steps). b) **24**, *n*-BuLi, $Me_2N(CH_2)_2OH$, *n*-hexane, 0 °C, then **31**, –30 °C to rt (57%). c) (i) Burgess reagent, toluene, 80 °C; (ii) $H_2$, 5% Pd-C, EtOH, rt; (iii) TBAF, THF, rt (36% in 3 steps). d) (i) $SO_3$·pyridine, $Et_3N$, DMSO, $CH_2Cl_2$, 4 °C to rt; (ii) $NaClO_2$, $NaH_2PO_4$, 2-methyl-2-butene, *t*-BuOH, $H_2O$, rt; (iii) $CH_3NH_2$, HATU, *N*,*N*-diisopropylethylamine, DMF, rt (47% in 3 steps). e) (i) **17**, CuI, $PdCl_2(PPh_3)_2$, $Et_2NH$, $PPh_3$, DMF, 120 °C; (ii) TBAF, THF, rt (36% in 2 steps). f) (i) $H_2$, Lindlar cat., quinoline, MeOH, rt; (ii) MsCl, $Et_3N$, $CH_2Cl_2$, –20 °C (37% in 2 steps).

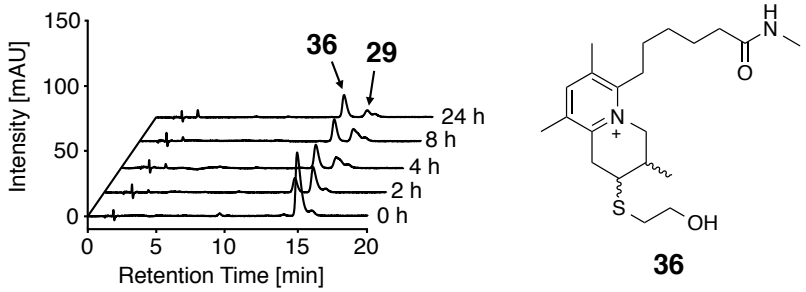



**Fig. 3.** HPLC chromatograms of the reaction mixtures of 2-mercaptoethanol with **29** (left) and the structure of adduct **36** (right).

## 3. Conclusion

In this study, thirteen analogs of QCD (**2**–**14**) were synthesized and evaluated for their cytotoxicity against HeLa-S3 cells and Michael acceptor reactivity toward 2-mercaptoethanol. The results collectively indicated that the thiol-trapping ability of QCD plays a marginal role in its cytotoxicity, and that the pyridinium moiety with the hydrophobic side chain is a key structural factor for cytotoxicity. Upon considering that quinolizinium and pyridinium compounds with long alkyl chains have been reported to induce cell membrane disruption and apoptosis-mediated cell death,[16] it is quite likely that the mode of cytotoxic action of QCD is similar to that of these cationic compounds. Of another significance, amide-containing analog **29** retained the Michael acceptor function without showing cytotoxicity. Given that various protein ligands could connect with the side chain of **29** through the amide linkage, the present result suggests the potential use of this compound for the design of covalent inhibitors and protein-labeling probes. In particular, an intriguing feature of the 3,4-dihydroquinolizinium ring is the presence of a positive charge, which may allow selective labeling of cysteine residues near negatively charged amino acid residues of the target proteins. Further investigation into the medicinal and biological applications of QCD in is underway, and the results will be reported in due course.

## 4. Experimental

### *4.1. Synthesis of **2**–**14** and **29***

Experimental details and characterization are provided in Supplementary material.

### *4.2. Thiol-trapping experiment with 2-mercaptoethanol*

To a solution of **2**, **11**, **14** or **29** (0.26 μmol) in PBS buffer (100 μL, pH 7.4) was added a solution of 2-mercaptoethanol (203 μg, 2.6 μmol) in PBS buffer (100 μL, pH 7.4). After stirring at room temperature for 0, 2, 4, 8, and 24 h, 5 μL of the reaction mixture was subjected to HPLC analysis (column: CAPCELL PAK C18 MG II S-5 column; solvent: $CH_3CN$/water 25→60% 20 min linear gradient for **2**, 35→60% 20 min linear gradient for **11** and **14**, 15% isocratic for **29**) containing 0.1% TFA; flow rate: 1.0 mL/min; UV detection: 300 nm for **2**, **11**, and **14**, 305 nm for **29**). Characterization of the adduct **36** is provided in Supplementary material.

### *4.3. Cytotoxicity test*

Cytotoxicity against HeLa-S3 (SC) cells (RIKEN BRC through the National Bio-Resource Project of the MEXT) and HEK293 cells (ATCC) was evaluated by following the method reported previously.[17] Briefly, HeLa-S3 (SC) and HEK293 cells were cultured in Eagle's minimal essential medium (EMEM) (Wako Pure Chemical Industries) and Dulbecco's modified Eagle's medium (DMEM) (Nissui), respectively, supplemented with 10% bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin (Thermo Fisher Scientific). A total of 10,000 cultured cells were seeded into each well of a 96-well plate (Thermo Fisher Scientific) containing 99 μL of the same medium. After preincubation for 24 h at 37 °C in an atmosphere of 5% $CO_2$, a test compound in 1 μL of DMSO was added to each well, and the cells were incubated for an additional 48 h. A solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (50 μg, Wako Pure

Chemical Industries) in PBS buffer (10 μL) was then added to each well, and the plate was incubated for an additional 3 h. Subsequently, the medium was removed by aspiration, any generated formazan was dissolved in 100 μL of DMSO, and the absorbance was measured at 595 nm using a Multiskan FC microplatereader (Thermo Fisher Scientific). $IC_{50}$ values were determined from the dose–response curves (for raw data, see Supplementary material).

**Declaration of Competing Interest**

The authors declare no conflict of interest.

**Acknowledgements**

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