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Structure–activity relationship studies on UK-2A, a novel antifungal antibiotic from *Streptomyces* sp. 517-02. Part 5: Roles of the 9-membered dilactone-ring moiety in respiratory inhibition

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Abstract—Several open-chained analogues of UK-2A, a novel antifungal antibiotic isolated from *Streptomyces* sp. 517-02, were prepared for structure–activity studies. The in vitro antifungal activities of these compounds against *Rhodotorula mucilaginosa* IFO 0001 and the inhibition of uncoupler-stimulated respiration in bovine heart submitochondrial particles (SMP) were evaluated. Oxidative potentials were measured by cyclic voltammetry. An analogue prepared from dihexyl L-glutamate showed comparable inhibitory activity as UK-2A.

UK-2A is an antifungal antibiotic produced by *Streptomyces* sp. 517-02 and is similar to antimycin A_3 (AA) in both structure and inhibitory activity toward electron transport at mitochondrial complex III.[1–4] Both UK-2A and AA consist of 9-membered dilactone rings linked via an amide bond to an aromatic acid moiety (Fig. 1): UK-2A possesses a 3-hydroxy-4-methoxypicolinic moiety, while the AAs have 3-formamidosalicylic moieties, which are reported to be essential for complex III binding and inhibition of electron transfer between cytochromes *b* and c_1 in the mitochondrial respiratory chain.[5] A benzyl group at the C2-position in UK-2A has recently been reported in antimycin A_9 as a phenylacetyl residue connected to C3 via an ester bond;[6] however, a methyl group is lacking at the C8-position. Furthermore, UK-2A was less cytotoxic than AA against several mammalian cell lines, including murine leukemia P388, murine melanoma B16, porcine renal proximal tubule LLC-PK_1, human oral epidermoid carcinoma KB, and human colon adenocarcinoma COLO201.[7] In our continuing studies on UK-2A,[8] we have focused on establishing the structure–activity relationships among UK-2A analogues.[9–12] We recently demonstrated that strict respiratory inhibition and the subsequent ROS generation does not directly induce

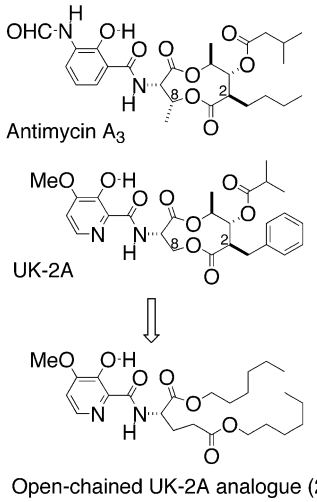

Figure 1. Structures of AA, UK-2A, and analogue **2**, showing the focus of SAR.

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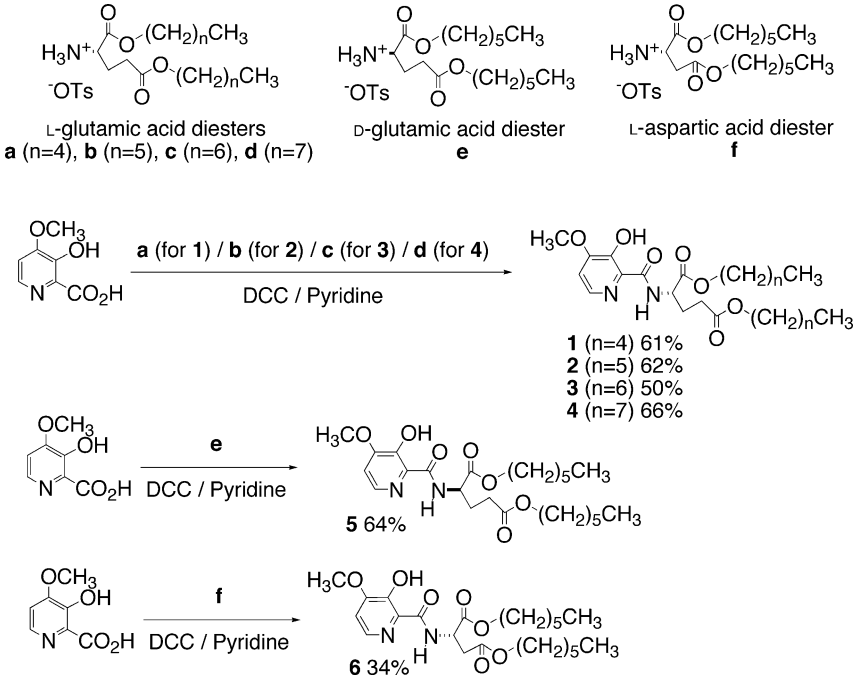
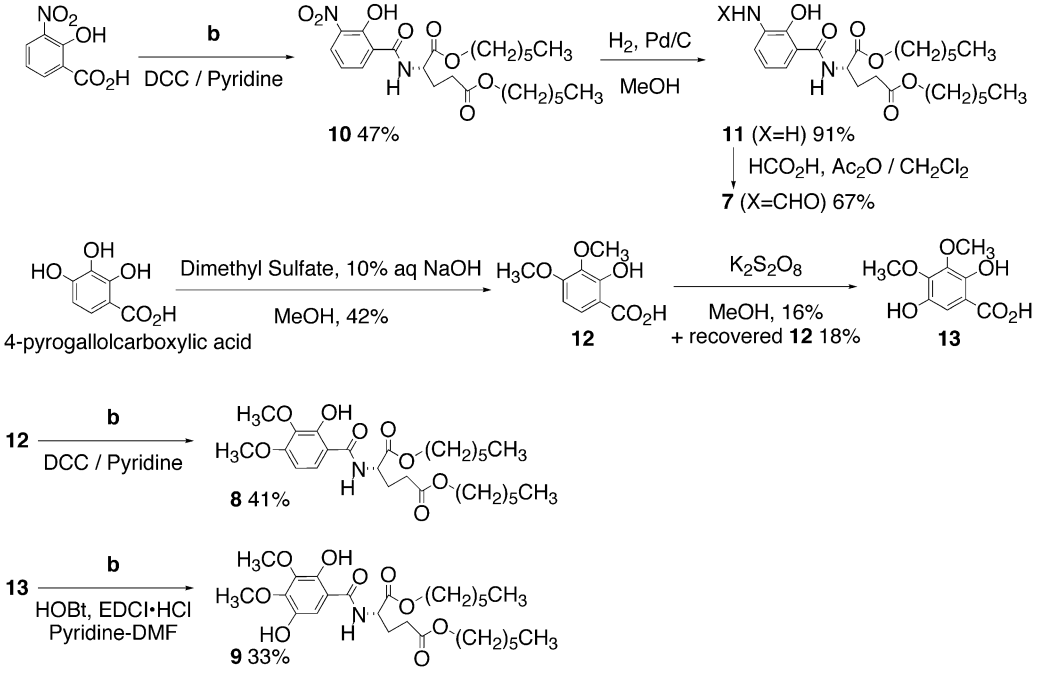
cytotoxicity and that the methyl group at the C8-position contributes to the cytotoxic activity of the AA derivatives.[12] This study focuses on the 9-membered dilactone ring itself, which was replaced by suitable mimetic structures, as shown in Figure 1, and reports the results of preliminary studies on the preparation and biological evaluation of open-chained UK-2A analogues.

In order to examine the role of the dilactone moiety in biological activity, UK-2A analogues **1**–**6** were prepared as shown in Scheme 1. Diesters of L-glutamic acid **a**–**d** were prepared from C5–C8 alcohols by conventional methods.[13] Amide formation with 3-hydroxy-4-methoxypicolic acid proceeded smoothly to afford the corresponding analogous compounds **1**–**4** in moderate yields. Compounds **5** and **6** were prepared from dihexyl D-glutamate **e** and dihexyl L-aspartate **f**, respectively, in the same manner. Preparation of **7**–**9** is summarized in Scheme 2. AA analogue **7** having a 3-formylaminosalicylyl group was prepared from 2-hydroxy-3-nitrobenzamide **10** in 2 steps: hydrogenation over 10% Pd on carbon in MeOH and N-formylation with HCO_2H-Ac_2O in CH_2Cl_2. Selective bis-methylation of 4-pyrogallolcarboxylic acid was achieved to afford 3,4-dimethoxysalicylic acid **12**.[14] Subsequent oxidation with $K_2S_2O_8$ provided the corresponding hydroquinone **13** in 16% yield.[15] Amide formation of **12** and **13** with **b** proceeded to afford ubiquinol analogues **8** and **9**, respectively, in moderate yields. The identity and purity of **1**–**9** were established by ^1H NMR and MS. On the ^1H NMR spectra (400 MHz, $CDCl_3$) of each compound, a signal for the phenolic OH proton was seen at 11.7–12.9 ppm. This downfield-shifted signal suggests the formation of an intramolecular hydrogen bond between the phenolic OH proton and the carbonyl oxygen of the salicylamide linkage. The intramolecular hydrogen bond was shown in the crystal structure of AA and a rearrangement of the hydrogen-bonding pattern upon binding was observed in the bovine mitochondrial bc_1 complex with AA bound.[16]

Compounds **1**–**9** were then evaluated for in vitro antifungal activity against a strict aerobic yeast, *Rhodotorula mucilaginosa* IFO 0001. For antifungal assay, each compound was first dissolved in DMF. Minimal inhibitory concentration (MIC) values were determined by the serial 2-fold agar dilution method[17] after 48 h of cultivation on Sabouraud dextrose agar at 25 °C. The results are summarized in Table 1. All tested UK-2A analogues (**1**–**6**), except **4**, inhibited the growth of *R. mucilaginosa* IFO 0001, although the inhibitory activities of **1**, **3**, **5**, and **6** were more than 100–1000 times less than that of UK-2A, demonstrating that the 9-membered dilactone moiety is not essential for such an weak antifungal activity, as reported for AA.[5] This is also consistent with our previous results.[9] The length of alkyl chain has some effect on antifungal potency; two *n*-hexyl groups could fit best in the ubiquinol pocket and provide suitable hydrophobicity for membrane permeability to a series of L-glutamic acid diester analogues. The decrease in inhibitory activity of D-glutamate **5** is attributable to a stereochemical mismatch that interferes with binding to the active site of complex III. The MIC of **6** was lower than that of **2**, thus suggesting that the distance between the two carbonyl groups affects growth inhibitory activity. Ubiquinol analogue **9** showed weak antifungal activity, although the reasons are not apparent. Among the derivatives evaluated here, *n*-hexyl diester **2** showed the strongest inhibitory activity; compound **2** was further compared with UK-2A in respiratory inhibition in addition to **4** and **7**–**9**.



Scheme 1. Preparation of open-chained UK-2A analogues.



Scheme 2. Preparation of AA analogue **7** and ubiquinol analogues **8** and **9**.

Table 1. Antifungal activity of **1**–**9**, UK-2A, and AA against *Rhodotorula mucilaginosa* IFO 0001

Compound	MIC (μg/mL)
1	12.5
2	3.13
3	50
4	>100
5	100
6	100
7	>100
8	>100
9	100
UK-2A	<0.1
AA	0.39

Table 2. Respiratory inhibition of **2**, **4**, **7**–**9**, UK-2A, AA, and C8-UK-2A in bovine heart SMP

Compound	pI_{50}
2	6.0
4	5.7
7	7.1
8	4.9
9	5.0
UK-2A	6.0
AA	7.4
C8-UK-2A	4.7[a]

[a] Ref. 9.

Inhibitory activity of **2**, **4**, and **7**–**9** against uncoupler-stimulated respiration of bovine heart submitochondrial particles (SMP) was then examined. Bovine heart SMP were prepared using the method of Matsuno-Yagi and Hatefi.[18] SMP respiration using 10 mM succinate as the respiratory substrate was measured with a Hanza-tech oxygen electrode at 25 °C. Table 2 lists the molar concentrations of inhibitors needed to halve the 2,4-dinitrophenol-stimulated respiratory rate of bovine heart. The log of the reciprocal of I_{50} is taken as the inhibitory index. Compound **2** showed relatively high inhibitory activity when compared with C8-UK2A, which was previously reported.[9] Analogues **2** and **7** showed comparably high inhibitory activity similar to UK-2A and AA. Comparison between **2** and **4** in respiratory inhibition suggests that the change of the hydrophobicity has little effect on the inhibitory activity. Measurement of oxygen consumption by bovine heart SMP in the presence of various respiratory substrates revealed the effect of **2** on SMP respiration; **2** inhibited the respiration using β-hydroxybutyrate as a substrate of mitochondrial complex I. After the addition of rotenone and succinate, the inhibition of respiration did not disappear. Moreover, no inhibition was observed using ascorbate-reduced tetramethyl *p*-phenylenediamine as the substrate. These indicate that the site of the respiratory inhibition of **2** is complex III of mitochondrial electron transport, as is the case for UK-2A. When the intact cell suspensions of *R. mucilaginosa* IFO 0001 were incubated with **2**, quick decrease in the respiratory activity was observed; addition of UK-2A or **2** at 3.13 μg/mL resulted in 50% inhibition at 30 min. These results suggest that the binding site conformation of the dihexyl L-glutamate moiety is similar to that of the 9-membered dilactone moiety in UK-2A and AA (ubiquinone reduction site of cytochrome bc_1 complex).[16] However, the antifungal activities of **2** and **7** were weaker than those of UK-2A and AA. This could be due to the limited cell membrane

Table 3. Oxidative potential of **2**, **7**–**9**, UK-2A, AA, and QH_2

Compound	Ep (mV)
2	+1125
7	+1141
8	+1119
9	+786, +1577
UK-2A	+1108
AA	+1057
QH_2	+810, +1402

permeability of **2** and **7**. The activities of **8** and **9** were about 1% of that of UK-2A, indicating that these ubiquinol analogues do not act as inhibitors of respiration, even if they permeate into cells. This suggests the possibility that ubiquinol analogues **8** and **9** serve as mediators of electron transport.

Furthermore, cyclic voltammetric measurements were performed with a BAS-50W electrochemical analyzer using a conventional three-electrode system, in which a glassy carbon disk (ϕ = 3 mm), an $Ag|Ag^+$ (0.01 M) acetonitrile, and a platinum electrode were the working, reference, and counter electrodes, respectively. The sample solutions (~0.2 mM) in acetonitrile containing 0.1 M Bu_4NPF_6 were degassed with Ar prior to the voltammetric measurements at room temperature. The ferrocene/ferrocenium redox couple was used as an internal standard and the redox potential of the couple was +200 mV under the above conditions. The voltammograms for compounds **2**, **7**–**9**, UK-2A, AA, and ubiquinol (QH_2)[19] show well-defined oxidation peak(s). The oxidation potentials are summarized in Table 3. Compounds **2**, **7**, **8**, UK-2A, and AA gave one oxidation peak at around +1100 mV and no significant correlations between oxidative potential and structure/MIC were observed. Compounds **9** and QH_2 had two oxidation peaks; the second oxidation potential of QH_2 (+1402 mV) was much lower than that of **9** (+1577 mV). This suggests that oxidation of **9** to a quinone derivative to mediate electron transfer does not proceed in vivo.

In summary, the 9-membered dilactone ring in UK-2A and AA could be substituted for open-chained L-glutamic acid esters with no effect on respiratory inhibition in SMP. However, antifungal activity depends on the structure or hydrophobicity of the dilactone ring, as cell membrane permeability is necessary.

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