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# Synthesis and biological investigation of new 4″-malonyl tethered derivatives of erythromycin and clarithromycin

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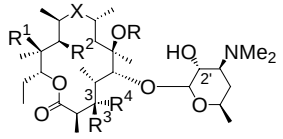
**Abstract**—A new approach to 4″-substituted derivatives of erythromycin and clarithromycin was developed by converting them into corresponding 4″-malonic monoesters. Subsequent carbodiimide coupling with alcohols and amines provided new macrolide derivatives that are capable of binding to 50S ribosomal subunits and inhibiting protein synthesis in cell-free system.


Erythromycin **1a** and other macrolide antibiotics (Scheme 1) have been used for the treatment of a variety of bacterial infections for the past 50 years. Low toxicity and cost as well as low incidence of side effects resulted in extensive use of erythromycin both for treatment of infections and prophylactic purposes.[1] The active use of the drug resulted in a wide spread of macrolide resistance in a number of pathogenic strains. As an example, in Asia the majority (up to 80% in Hong Kong) of *Streptococcus pneumoniae* strains carry resistance to macrolide antibiotics.[2] Attempts to improve relatively poor bioavailability of erythromycin, increase its spectrum of activity, and overcome bacterial resistance resulted in synthesis and investigation of a number of semisynthetic macrolide derivatives such as clarithromycin **1b**, azithromycin **1c**, and roxithromycin **1d**. The most recent generation of macrolide antibiotics, such as telithromycin (KETEK®) **1e** and ABT-773 **1f**,[3] possesses enhanced biological activity; however, their synthesis is much more complicated.

All macrolide antibiotics share a similar mechanism of action which involves selective binding to the 50S subunit of bacterial ribosome resulting in inhibition of protein synthesis. Genetic, biochemical, and more recent crystallographic studies of ribosome–macrolide complexes have indicated that macrolides bind in nascent peptide exit tunnel thus blocking polypeptide chain growth.[4a–i] X-ray structures showed that desosamine sugar at position 5 of the macrolide lactone ring provides very important contribution to the overall binding energy of the drug.[4a,i,5] Indeed, acylation of 2′-hydroxy group of desosamine drastically decreases antibacterial activity.[6] Newer macrolide derivatives such as telithromycin, ABT-733 or bridged ketolides possess additional heterocyclic arm which is most likely responsible for their enhanced antibacterial activity.[3]

The extent of involvement of the cladinose sugar in ribosome binding is less clear. While a simple removal of the cladinose moiety was found to substantially decrease the activity of erythromycin,[7] a number of highly active macrolide derivatives, such as acylides[8] and ketolides,[9]



**1a** R=H, $R^1$=$R^2$=OH, $R^3$=H, $R^4$= cladinose, X= C=O
**1b** R=Me, $R^1$=$R^2$=OH, $R^3$=H, $R^4$= cladinose, X= C=O
**1c** R=H, $R^1$=$R^2$=OH, $R^3$=H, $R^4$= cladinose, X= N(Me)$CH_2$
**1d** R=H, $R^1$=$R^2$=OH, $R^3$=H, $R^4$= cladinose, X= C=N$OCH_2OCH_2CH_2OMe$
**1e** R=Me, $R^1$,$R^2$= -OC(O)N($CH_2$)$_3$–N(=N)–3-Pyridyl, $R^3$, $R^4$=O, X= C=O
**1f** R=$CH_2CH$=CH-3-Quinolinyl, $R^1$,$R^2$= -OC(O)NH- , $R^3$, $R^4$=O, X= C=O

**Scheme 1.** Erythromycin and semisynthetic macrolide antibiotics.

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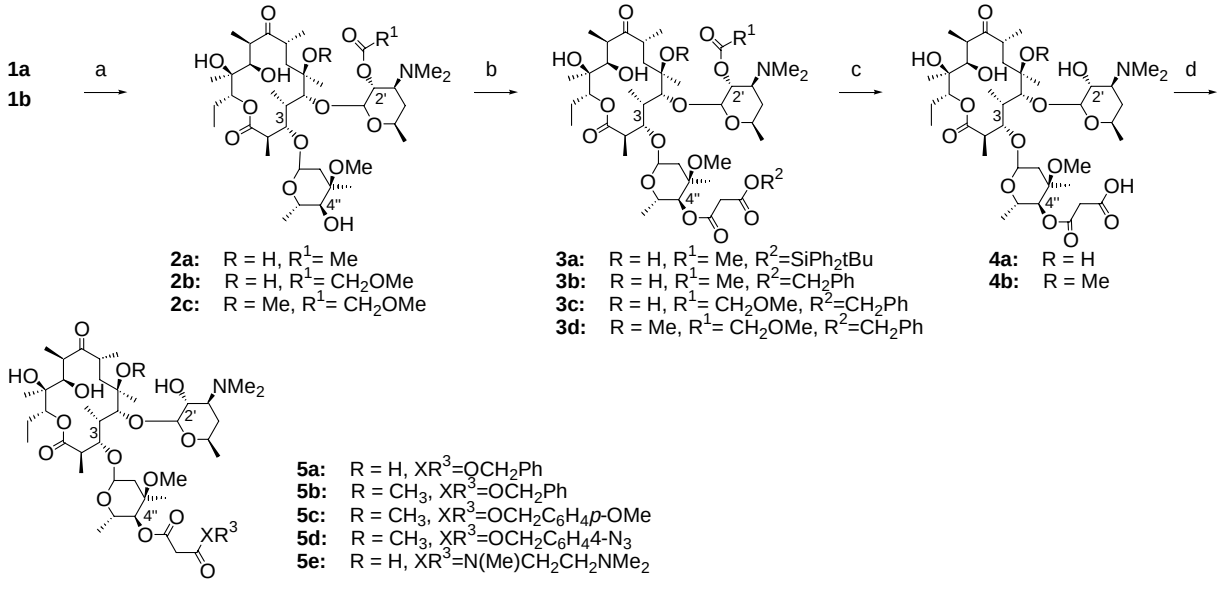
do not contain cladinose. There were relatively few attempts to modify cladinose residues to increase the bioavailability or activity of erythromycin. Attempts to introduce simple alkanoyl[6,10] and polyunsaturated alkenoyl[11] substituents into 4″ position of erythromycin involved either diacylation at 2′ and 4″ positions followed by a selective hydrolysis, or selective formylation of 2′-position followed by the acylation of 4″-position and selective hydrolysis of 2′ formyl group.[6] Resultant compounds exhibited lower[10] or substantially lower[12] than erythromycin antibacterial activity,[13] most probably due to decreased bioavailability. High nucleotide content of ribosome gives rise to a number of possible interactions such as hydrogen bonding, π-stacking as well as electrostatic interactions. Erythromycin and clarithromycin derivatives carrying cladinose sugar modified with functional groups capable of such types of interaction have a potential to exhibit increased affinity toward ribosomes including mutant ribosomes of resistant bacteria. Indeed, Fernandes and co-authors demonstrated substantial enhancing of antibacterial activity through alteration of 4″-position of erythromycin oxime derivatives with heteroatom-functionalized side chains.[14] This type of derivatization, however, is relatively difficult and laborious to perform by previously described methods.[6]

We recently reported on a new one-pot method for tethering of organic molecules through the formation of non-symmetric malonate derivatives.[15] This method enables efficient binding of alcohols and amines under very mild conditions in the absence of acylation catalysts. Since malonate linker is biocompatible and reasonably stable, we were attracted by the possibility to derivatize cladinose sugar of macrolide antibiotics by introducing functional groups that could influence binding of the drug to the ribosome. In contrast to all previous attempts for selective 4″-derivatization of macrolides, our synthetic approach to 4″-tethered macrolide derivatives of type **5** (Scheme 2) involved the use of one common intermediate of type **4**.

In line with this approach, 2′-acetyl erythromycin **2a** was prepared by a treatment of **1a** with acetic anhydride in the absence of acylation catalysts.[16] No acylation of other hydroxyl groups was detected even in the presence of a large excess of acetic anhydride. This is possible due to the vicinity of $NMe_2$ group in desosamine residue which substantially increases rates of both introduction and removal of 2′-acyl group.[6] In contrast, acylation of **1a** with an excess of acetic anhydride in the presence of DMAP yielded a complex mixture in which products possessing one to four acetyl groups were detected by ESI-MS. Compound **2a** was then acylated by DCC coupling through non-symmetric malonate method[15,17] using *tert*-butyldiphenylsilyl and benzyl malonates. The reaction of 2′-acetyl erythromycin **2a** with *tert*-butyldiphenylsilyl malonate and DCC was found to provide a mixture of starting material, mono-, and diacylation products. In contrast, acylation of **2a** with benzyl malonate/DCC system proceeded smoothly to give benzyl ester **3b** without diacylation. Reasons for such a different reactivity are not clear since *tert*-butyldiphenylsilyl malonate was found to be less reactive than benzyl malonate in acylation of other sterically hindered alcohols. The structure of **3b** was verified by the ESI MS–MS technique. Fragmentation of $MH^+$ of **3a** produced $MH^+$-cladinose-$C(O)CH_2CO_2Bn$ cations. No traces of $MH^+$-cladinose ions that would indicate the presence of isomers other than 4″-benzylmalonylerythromycin were observed.

We initially planned to remove the 2′-acyl group by methanolysis of ester **3b**. However, there was a considerable decrease in the methanolysis rates of malonyl derivative **3b** in comparison to 2′-acetyl erythromycin **2a**




**Scheme 2.** Preparation of cladinose tethered derivatives of erythromycin and clarithromycin. Reagents and conditions: (a) $(R^1CO)_2O$, DCM, rt; (b) $R^2OCOCH_2CO_2H$, DCC, rt; (c) MeOH, rt, 24 h, then $H_2$/Pd/MeOH/acetate buffer; (d) $HXR^3$, DCC, DCM, rt.

which resulted in competing methanolysis of 4″-benzylmalonyl functions thus contaminating the desired 2′-deacetylated product **4a** with erythromycin. A number of alternative 2′-protecting groups were evaluated. Acylation of erythromycin with anhydrides of chloroacetic and dichloroacetic acids as well as $HCO_2COMe$ failed to provide the desired 2′-acylation products. In contrast, reaction with methoxyacetic anhydride prepared by the treatment of methoxyacetic acid with DCC was found to proceed smoothly producing 2′-methoxyacetyl erythromycin **2b**. As expected, 4″-acylation of **2b** also proceeded selectively thus providing diester **3c** that after subsequent methanolysis and catalytic hydrogenation provided acid **4a** without impurities of erythromycin. The same synthetic sequence was successfully applied to clarithromycin to afford acid **4b**.

Since carboxylic groups of malonates **4a**,**b**, as those of other malonate monoesters, are capable to form ketene intermediates upon the treatment with carbodiimides, several new macrolide derivatives were prepared by carbodiimide-mediated coupling with compounds possessing hydroxy and/or amino groups. DCC-mediated coupling of **4a**,**b** with benzyl, 4-methoxybenzyl, and 4-azidobenzyl alcohols provided corresponding non-symmetric malonates **5a**–**d**. A reaction with trimethylethyl enediamine was found to proceed much slower and the use of two equivalents of the amine and DCC was essential to achieve 35% isolated yield.

Compounds **4a**,**b** and **5a**,**b**,**e** were tested for their ability to inhibit the growth of a macrolide-sensitive *Escherichia coli* strain HN818 that lacks AcrAB transporter.[18] Minimal inhibitory concentrations of control drugs, erythromycin and clarithromycin, were 2 μg/ml. MIC of **5a**,**b**, and **e** were somewhat higher—8 μg/ml, whereas MIC of **4a**,**b** were significantly higher—128 μg/ml.

Activity of compounds **4a**,**b**, **5a**,**b**,**e** as protein synthesis inhibitors was tested in *E. coli* cell-free translation system. Though inhibitory activity of compounds **4a** and **4b** was reduced compared to that of erythromycin ($IC_{50}$ 3.7 and 3.9 μM vs 0.9 μM), inhibition of translation afforded by compounds **5a**, **5b**, and **5e** (1.1, 1.2, and 1.1 μM correspondingly) was comparable to that of erythromycin. Given efficient inhibition of cell-free protein synthesis by compounds **5a**,**b**, and **e**, we can conclude that their somewhat reduced antibacterial activity (MIC 8 μg/ml vs 2 μg/ml) should be attributed to their decreased uptake by the cell, possibly due to increased molecular weight. High in vitro activity of compounds **5a**,**b** possessing bulky and relatively rigid benzyl groups indicates that the ribosome can accommodate fairly large substitutions at this position—an observation compatible with the available crystal structures.

Since steric effects do not appear to play substantial role in ribosome binding, the lower activity of acids **4a**,**b** should be attributed to either hydrophobic environment in binding region or, possibly, the presence of a phosphate group in a close vicinity to the carboxylate function in the ribosome complexes with either **4a** or **4b**. The presence of a negatively charged group obviously significantly reduced uptake of the compounds **4a**,**b** by the cell reflected in their high MICs.

Interaction of several new compounds with the ribosome was further studied using RNA footprinting technique.[4g] Ribosomes were complexed with erythromycin or telithromycin (as controls) or with one of the newly synthesized compounds: **4a**, **4b** or **5e**, and rRNA was modified with dimethyl sulfate (DMS). Desosamine sugar of active macrolide antibiotics is known to protect adenines at positions 2058 and 2059 of 23S rRNA from DMS modification.[4g] Both of the control drugs, erythromycin and telithromycin, protected these rRNA positions from DMS modification. Similarly, the tested compounds with derivatized cladinose sugar effectively protected A2058 and A2059 from modification thus confirming binding of the new derivatives at the macrolide site. Thus, increased bulkiness of the substitution at position 3 of lactone ring does not prevent new derivatives from binding to the 'conventional' macrolide site in the ribosome. In *E. coli* ribosome, the extended side chain of telithromycin reaches across the nascent peptide exit tunnel and interacts with the loop of helix 35 of 23S rRNA where it protects the adenine residue at position 752 of 23S rRNA from modification with DMS. In contrast, binding of erythromycin or clarithromycin slightly enhances accessibility of A752 to modification with DMS.[5,19] Neither of the three tested compounds, **4a**,**b** or **5e**, protected A752. Instead, a slight enhancement of its modification with DMS, similar to that seen in the presence of erythromycin, was observed (data not shown). No other protection or enhancement in modification of 23S rRNA nucleotides was seen. Thus, we conclude that in contrast to the side chain of ketolide antibiotics, the side chains at the cladinose sugar do not reach position 752, suggesting that its orientation is different from that of the ketolide's alkyl-aryl extension. Given that in the currently available crystallographic structures of the ribosome–macrolide complexes, the cladinose sugar points in the general direction of the peptidyl transferase center, we anticipate that upon further growth of the side chain it will eventually reach positions 2610 or 2555 located at the peptidyl transferase/exit tunnel doorway.

In conclusion, we developed a new common precursor approach to the synthesis of 4″-derivatized macrolide derivatives and tested the resultant compound in several biological assays. Introduction of non-polar moieties into 4″-position was found to retain ribosome affinity but decrease bioavailability, while the introduction of acidic groups substantially decreases both of them.

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