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Synthesis and biological evaluation of novel bisheterocycle-containing compounds as potential anti-influenza virus agents

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Abstract—A series of novel 4,2-bisheterocycle tandem derivatives consisting of a methyloxazole and thiazole subunit were synthesized. Many compounds were found to inhibit human influenza A virus. Several analogues exhibited moderate biological activity and could serve as leads for further optimizations for antivirus research.

During the course of a search for bioactive metabolites from marine microorganisms, a variety of chemically interesting and biologically significant secondary metabolites containing heterocyclic tandem pairs consisting of oxazole and thiazole subunits were reported.[1] These products display a wide range of biological activities, such as antifungal, antileukemic, antimitotic, antibacterial, and gene-regulating properties, and inspired strong interest among many medicinal and synthetic chemists.[2] It was also reported that the heterocyclic tandem pair was possibly responsible for the biological activity,[3] such as that seen with the three consecutive oxazoles in Kabiramide C, which interfere with actin filament dynamics by binding to subdomains 1 and 3 of G-actin in an irreversible manner;[4] and the antibiotic Bleomycin, which interacts with DNA by binding of its bithiazole moiety to the nucleic bases in a mode that is partially intercalative, allowing penetration of the positively charged moieties distal to the bithiazole into the major groove.[5] Therefore, heterocyclic tandem pairs may be useful scaffolds in combinatorial libraries for new lead discoveries. Leucamide A is a bioactive cyclic heptapeptide from the Australian marine sponge *Leucetta microraphis*, containing a unique mixed 4,2-bisheterocycle tandem pair consisting of a methyloxazole and a thiazole subunit, and which was found to be moderately cytotoxic toward several tumor cell lines.[6]

Although Leucamide A itself does not show any antiviral activity, the unique mixed 4,2-bisheterocycle tandem pair in Leucamide A provides us with a useful scaffold for searching for potentially therapeutic compounds, especially antiviral agents, because the heterocyclic tandem pair may provide bioactivity through specific interactions with DNA/RNA. Following our first total synthesis of Leucamide A,[7] we constructed a library of 4,2-bisheterocycle tandem derivatives consisting of a methyloxazole and a thiazole subunit. Here, we report on the design and synthesis of a library of novel compounds containing 4,2-bisheterocycle tandem pairs and their inhibitory effects on influenza A virus in vitro and a preliminary study of their structure–activity relationships (Fig. 1).

Two series of compounds, **2a**–**2n** and **3a**–**3e**, were designed with various substituents, as shown in Figure 1. Synthesis of the compounds was as shown in Scheme 1. Compound **4** was synthesized as a key intermediate for the combinatorial generation of **2a**–**2n** and

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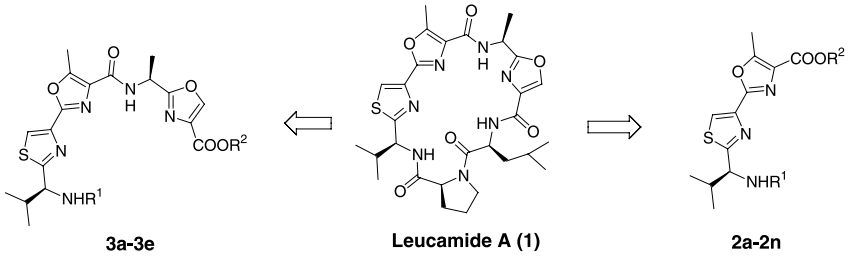
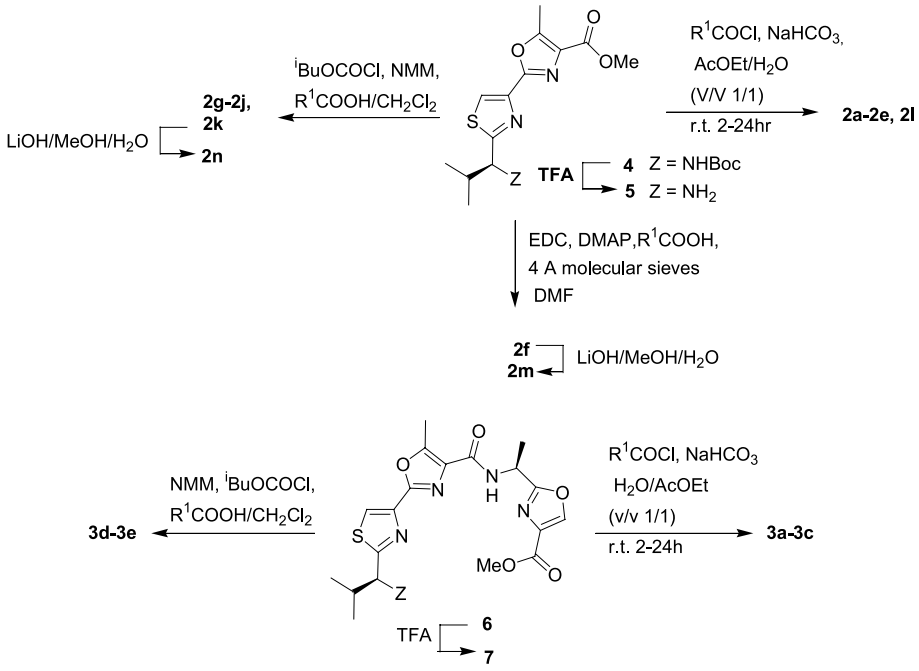


Figure 1. A focused library of 4,2-bisheterocycle tandem derivatives consisting of a methyloxazole and thiazole subunit.



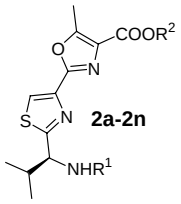
Scheme 1. Synthesis of 4,2-bisheterocycle tandem derivatives **2a**–**2n** and **3a**–**3e**.

3a–**3e**. Therefore, the initial goal was to produce gram quantities of **4** and **6**[7] suitable for more in-depth in vitro studies with the flexibility to produce analogues for exploration of structure–activity relationships. Compounds **2a**–**2n** were prepared as outlined in Scheme 1. The N-Boc-protecting group of **4** was removed using trifluoroacetic acid (TFA) and the resulting amine **5** was treated with acyl chloride to afford **2a**–**2e** and **2l** (Method A)[8] or reacted smoothly with the corresponding acid to give **2g**–**2k** (Method B)[8] or **2f** (Method C).[8] Compounds **3a**–**3e** were obtained by the method shown in Scheme 1. Compound **6** was transformed into compound **7** by deprotection of the N-Boc group with CF_3COOH–CH_2Cl_2, followed by amidation with acyl chloride to afford **3a**–**3c** (Method A) or with the corresponding acid to give **3d**–**3e** (Method B). Saponification of **2j** and **2l** with lithium hydroxide in aqueous methanol gave the corresponding carboxylic acids, **2n** and **2m**, respectively.

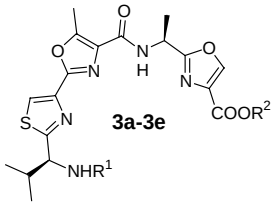
A series of derivatives were evaluated for their ability to inhibit influenza A virus H3N2 (A3 China/15/90) replication in Madin–Darby canine kidney (MDCK) cells.[9] The results are summarized in Table 1. Several compounds (**2i**, **2l**–**2n**) showed moderate activity against influenza A virus, with IC_{50} values of 29–37 μg/mL. Acid derivatives (**2m**, **2n**) were about twofold more active than corresponding methyl ester derivatives (**2f**, **2k**). These results suggested that the hydrophilic effects of a carboxyl group might lead to enhancement of activity, although no definitive explanations for these observations have yet been obtained. Among compounds **2a**–**2d** bearing various R^1 substituents on NH, the compounds with the large side chain (cyclohexanecarbonyl group) showed better inhibitory activity than those with small ones, and these results indicated that steric factors influence inhibitory activity. Increasing side-chain size from hydrogen (**5**) and propionyl (**2a**) to cyclobutanecarbonyl (**2b**) and large groups (**2c**, **2d**) has a clearly advantageous effect on antiviral activity. A twofold increase in antiviral activity was observed for 2-methoxy-benzoyl derivatives (**2f**) compared to 3-fluoro-benzoyl derivatives (**2e**), underscoring the importance of having an electron-donating group on the phenyl.

Among compounds **2g**–**2l**, containing unsaturated aliphatic carbonyl substituents on the NH, the 4-methyl-pent-3-enoyl derivative (**2g**) exhibited low activity against influenza A virus, with IC_{50} values of 192.45 μg/mL. With additional methylene spacers between the double bond and carbonyl group, the activity

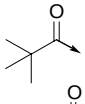
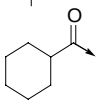
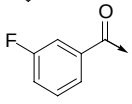
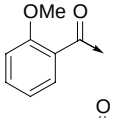
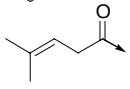
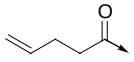
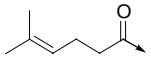
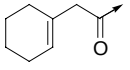
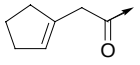
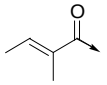
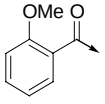
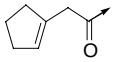
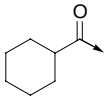
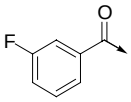
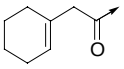
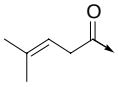
Table 1. Anti-influenza virus A activity and cytotoxicity of 4,2-bisheterocycle tandem derivatives in MDCK cells[a]



2a-2n



3a-3e

Compound	R^1	R^2	CC_{50}[b] (μg/mL)	IC_{50}[c] (μg/mL)
2a		Me	577.35	258.68
2b		Me	500	111.11
2c		Me	577.35	97.81
2d		Me	144.34	43.58
2e		Me	>1000	>86.23
2f		Me	333.33	47.72
2g		Me	>1000	192.45
2h		Me	577.35	258.69
2i		Me	577.35	37.03
2j		Me	231.12	68.71
2k		Me	333.33	64.15
2l		Me	231.12	37.03
2m		H	333.33	29.01
2n		H	480.75	29.01
3a		Me	577.35	160.25
3b		Me	144.34	57.78
3c		Me	577.35	77.04
3d		Me	577.35	44.48
3e		Me	577.35	111.11
5	H	Me	577.35	258.69
Leucamide A			NA	NA
Ribavirin			>2000	3.73

[a] Abbreviations and strains used: MDCK, Madin–Darby canine kidney cells, influenza A H3N2 viruses (A3 China/15/90).
[b] Concentrations that cause microscopically detectable toxicity in virus-infected cultures.
[c] Concentrations required to reduce virus-induced CPE in MDCK cells by 50%.

of **2i** increased approximately sixfold. Deletion of two terminal methyl groups from **2i**, to give the corresponding compound **2h**, resulted in an eightfold decrease in antiviral activity. Restricting the unsaturated alkyl chain within a ring (**2j**, **2k**) led to a threefold increase of antiviral activity when compared with the straight chain analogue (**2g**). Compound **2l** showed similar potency when compared to compound **2i**.

In the case of the bisheterocycle tandem derivatives bearing one more oxazole unit (**3a**–**3e**), **3b**–**3d** were more potent than the propionyl derivative (**3a**). These results also indicated that introduction of a big hydrophobic group might lead to enhancement of activity. Compounds **3a**–**3e** showed similar activity to their corresponding analogues **2a**, **2d**, **2e**, **2j**, and **2g**. These results suggest that addition of an oxazole ring to the bisheterocycle tandem pair made no difference to the potency of the antiviral activity.

The natural product Leucamide A was inactive against influenza A virus, indicating that the restricted ring of the 4,2-bisheterocycle tandem pair dramatically reduces antiviral activity. Conversely, open chain 4,2-bisheterocycle tandem derivatives showed biological activities, which may imply an advantage of a rather flexible side chain, accommodating favorable conformation(s) for bioactivity. In addition, all compounds showed no activity against influenza B virus. Most compounds showed low cytotoxicity with the exception of **2d** and **3b**, which nonetheless still exhibited a reasonable selectivity index (CC_{50}/IC_{50}).

These compounds containing a 4,2-bisheterocycle tandem pair were moderately effective against human influenza A and may give us an opportunity to attain novel potent anti-influenza agents to overcome drug-resistant mutants. However, the molecular target of these 4,2-bisheterocycle tandem derivatives is unknown at present.

In conclusion, by a discovery process inspired by a natural product, we have uncovered a novel and moderate line of potential antiviral agents. These results suggest that the 4,2-bisheterocycle tandem pair probably plays a major role in the potent antiviral activity. These compounds could certainly serve as leads for the development of de novo antiviral agents. Further studies on their structure–activity relationships, optimization of these compounds, and identification of the molecular targets of these compounds, as well as exploration for new bisheterocycle tandem pairs, are actively underway in our laboratory.

Acknowledgments

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8. Typical procedure for preparation of compounds **2** and **3**. Method A: To a solution of amine (0.1 mmol) in 3 mL of a 1:1 EtOAc/H_2O mixture was added excess $NaHCO_3$ (160 mg, 1.9 mmol). The appropriate acid chloride (0.15 mmol) was added at 0 °C. The mixture was stirred for 2–24 h with gradual warming to room temperature. The reaction mixture was diluted with EtOAc (5 mL) and H_2O (2 mL). The aqueous phase was extracted with EtOAc. The combined organic phases were then processed in the usual way and chromatographed to yield the desired products. Method B: To a stirred solution of appropriate acid (0.1 mmol) in dry CH_2Cl_2 (2 mL) under N_2 at 0 °C were added *N*-methylmorpholine (NMM) (0.13 mmol) and isobutyl chloroformate (0.11 mmol). After 10 min, the solution of amine in dry CH_2Cl_2 (2 mL) was added, and then the solution was stirred for 1–24 h with gradual warming to ambient temperature. The mixture was partitioned between H_2O (20 mL) and EtOAc (20 mL). The phases were separated and aqueous phase was extracted with EtOAc (4×10 mL). The combined organic phases were then processed in the usual way to yield the desired products. Method C: To **5** (24 mg, 0.08 mmol) and 4 Å molecular sieves in DMF (1 mL) at −10 °C were added HOBT (27.0 mg, 0.2 mmol) and 2-methoxybenzoic acid (13.0 mg, 0.088 mmol), and the resulting mixture was stirred at −10 °C for 20 min, then EDCI (16.0 mg, 0.088 mmol) was added and the mixture was stirred for 2 h with gradual warming to room temperature. The reaction mixture was diluted with EtOAc (50 mL) and H_2O (20 mL). The aqueous phase was extracted with EtOAc (4×20 mL). The combined organic phases were then processed in the usual way and chromatographed to yield **2f** (29 mg, 86%).
9. MDCK cells were grown as specified in Eagle's minimum essential medium with 10% heat-inactivated fetal bovine serum (FBS) plus antibiotics (penicillin, 100 U/mL; streptomycin, 100 U/mL). Influenza A H3N2 viruses (A3 China/15/90) were propagated in the allantoic cavities of 10-day-old embryonated eggs. Virus titers were determined by hemagglutinin titration, according to standard procedures. Confluent MDCK monolayers were infected with Influenza A viruses for 2 h at 37 °C, after which the viral inoculum was removed and cells were treated with different concentrations of compound. When CPE result of the viral control group reached 4+, the result of compound treated group was observed. The dilution that gives 50% cytopathic effect was determined by the interpolating procedure of Reed and Muench.