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SYNTHESIS AND EVALUATION OF HAPALOSIN AND ANALOGS AS MDR-REVERSING AGENTS

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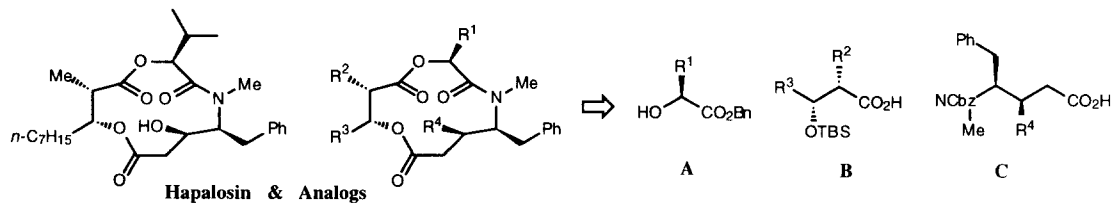
Abstract: The marine natural product hapalosin and 22 analogs, which incorporated systematic substituent deletions or variations, were prepared. These compounds were evaluated in a cell-based assay for both MDR-reversing activity and general cytotoxicity. Some substituent modifications resulted in lower cytotoxicities, but most structural changes were either detrimental to or did not seriously alter the MDR-reversing activity.

Introduction: One of the problems associated with the chemotherapeutic treatment of some cancers is the ability of the tumor cells after exposure to a cytotoxic drug to develop resistance to other unrelated drugs. This scenario is termed multidrug-resistance (MDR), and is one of the most serious obstacles facing the development of new chemotherapeutic agents and strategies for the treatment of cancers.[1] There is strong evidence to suggest that the phenomenon of MDR is mediated to a large degree by the activation or over-expression of P-glycoprotein (P-gp), which is a transmembrane protein acting as an ATP-dependent drug efflux pump. Enhanced efflux results in a reduction of drug accumulation in the cells with a concomitant reduction in drug-mediated cytotoxicity.

Antagonists of P-gp activity may be useful in combination therapy with cytotoxic drugs, and a continual search for such compounds has identified a number of naturally occurring and synthetic agents that can antagonize P-gp mediated MDR.[2] Unfortunately, progress in this area has been hampered by the lack of potent and nontoxic compounds.[3] Recently, the marine natural product hapalosin (Figure 1) was isolated and characterized as an MDR-modulator of novel structure, and was reported to show better MDR-reversing activity in vitro than verapamil.[4] Hapalosin appeared to be a promising candidate for medicinal chemistry efforts exploring the SAR of both MDR-reversing activity and general toxicity, and we now report the results from our investigation into the preparation of a series of hapalosin analogs and their in vitro evaluation as potential MDR-reversing agents.

Synthesis of hapalosin and analogs: A number of syntheses of hapalosin as well as some analogs have been published.[5] We prepared our compounds loosely following the synthetic plans of Ghosh and Zhu,[5b,c] utilizing well-known chemistries for the requisite intermediate fragments **A**, **B**, and **C**.



Hapalosin & Analogs **A** **B** **C**

The esters **A** were either commercially available (R^1 = H) or prepared from the carboxylic acids by treatment with BnBr/DBU/CH_3CN. The β-silyloxyacids **B** were prepared via Evans' oxazolidinone-based aldol chemistry[5b,6] as

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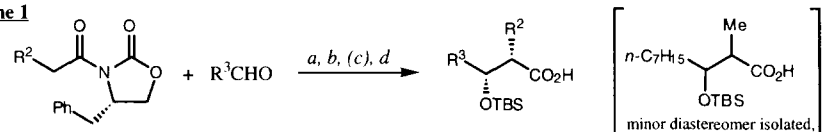
outlined in Scheme 1. The fragment with R^2=H, R^3 = n-C_7H_{15} was prepared from (methylthio)acetic acid with subsequent removal of the MeS-group (R^2) via Ra-Ni reduction. For the case R^2 = Me, R^3 = n-C_7H_{15}, a second aldol diastereomer was isolated as a minor product (stereochemistry undetermined) and was also used for two analogs. Silylation of (S)-lactic acid gave the fragment with R^2=Me, R^3=H.

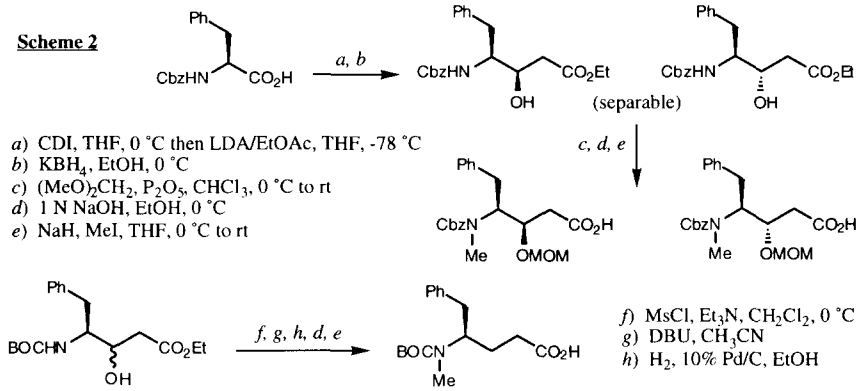
Scheme 1



a) $BuB(OTf)_2$, $EtNi\text{-}Pr_2$, CH_2Cl_2, -78 °C to 0 °C
b) TBSOTf, 2,6-lutidine, CH_2Cl_2, 0 °C
c) Ra-Ni, EtOH, rt (R^2= SMe → H); *d*) LiOH, 35% H_2O_2, THF, 0 °C

The protected amino acids **C** were readily prepared from phenylalanine following the work of Joullié,[7] as shown in Scheme 2. In our hands, the MOM-group was best installed using dimethoxymethane and P_2O_5 ($CHCl_3$, 0°C),[8a] as use of MOMBr and Hünig's base[8b] failed to give complete conversion of the starting materials. The deoxy fragment was prepared via a mesylation/elimination/hydrogenation sequence starting from a mixture of the 2°-alcohol diastereomers, which in turn were derived from *N*-BOC-phenylalanine.[7]

Scheme 2

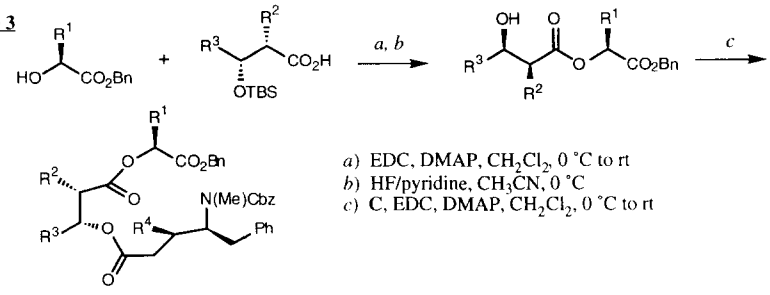


a) CDI, THF, 0 °C then LDA/EtOAc, THF, -78 °C
b) KBH_4, EtOH, 0 °C
c) $(MeO)_2CH_2$, P_2O_5, $CHCl_3$, 0 °C to rt
d) 1 N NaOH, EtOH, 0 °C
e) NaH, MeI, THF, 0 °C to rt

f) MsCl, Et_3N, CH_2Cl_2, 0 °C
g) DBU, CH_3CN
h) H_2, 10% Pd/C, EtOH

The fragments **A** and **B** were coupled using EDC and DMAP (Scheme 3), and the silyl ether was cleaved with HF/pyridine in CH_3CN.[5b] These 2°-alcohols were used to esterify the acids **C**, again under EDC/DMAP conditions, to provide the protected macrocyclization precursors.

Scheme 3

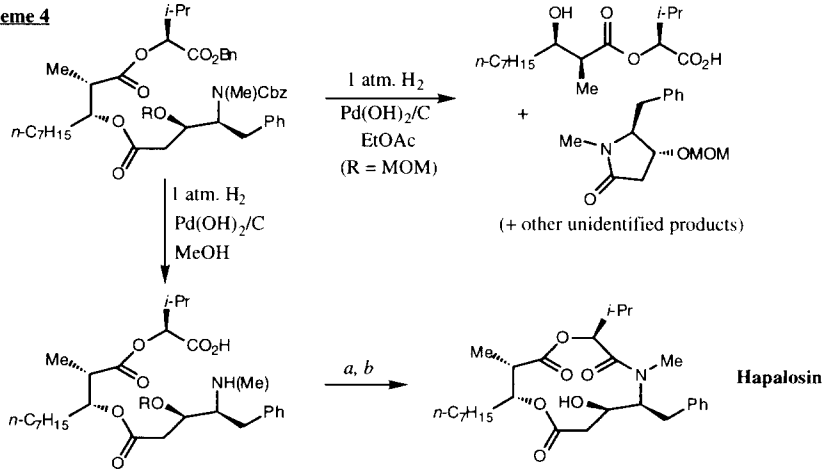


a) EDC, DMAP, CH_2Cl_2, 0 °C to rt
b) HF/pyridine, CH_3CN, 0 °C
c) C, EDC, DMAP, CH_2Cl_2, 0 °C to rt

Initial attempts at deprotection and macrocyclization to generate hapalosin (Scheme 4) were frustrated by the observation that the free amino acid underwent internal lactamization and fragmentation when the hydrogenation was carried out in EtOAc.[5c] When the solvent was changed to MeOH,[9] the hydrogenation was rapid (ca. 2 h) and

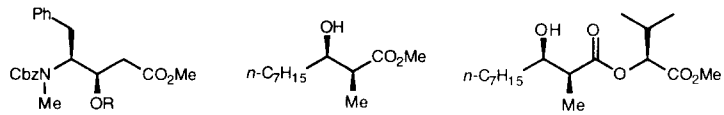
very clean, and the amino acid appeared to be quite stable. Cyclization of the crude material under Yamaguchi conditions (toluene, rt)[9] followed by cleavage of the MOM-ether[10] provided synthetic hapalosin. We do not have a suitable explanation for the solvent effect in the hydrogenolysis reaction, but for the preparation of analogs **1-22** (Table 1), methanol consistently gave a high yield of clean amino acid that could be purified (RP-HPLC) or simply carried on directly to the macrocyclization.

Scheme 4



a) 2,4,6-trichlorobenzoylchloride, DMAP, EtN*i*-Pr$_2$, toluene, rt
b) TMSBr, sieves, CH_2Cl_2

All of the macrocycles containing the MOM-group were submitted for in vitro testing as well as the materials with a free 2°-hydroxyl. The fragments **23-26** were prepared via direct methods from available intermediates and were also tested in vitro to examine their possible contributions to MDR-reversing activity as well as cytotoxicity.



23 R = MOM
24 R = H

25

26

Biological evaluation of hapalosin and analogs: MDR-reversing activities of hapalosin and analogs were evaluated using P388/VMDRC.04 cells, a subline of P388 murine leukemia cells expressing human recombinant human P-gp.[11] Briefly, P388/VMDRC.04 cells were grown for 3 days with 0-100 μM test compounds or verapamil, with or without 10 nM vincristine, an empirically-determined concentration that inhibits P388/VMDRC.04 growth only in the presence of an MDR-reversing agent such as 10 μM verapamil (P388/VMDRC.04 cells are normally resistant to 10 nM vincristine). After 3 days, cell growth was determined by MTT assay.[12] Verapamil was run in all assays as an internal control. MDR-reversing activities of test compounds were quantified as IC_{50} values by reappearance of vincristine's growth inhibitory effects. Cytotoxicity was quantified as IC_{50} values in the absence of 10 nM vincristine; and the MDR to cytotoxocity window calculated as IC_{50}[cytotoxicity]/IC_{50}[MDR-reversal].

Table 1. In Vitro Evaluation of Hapalosin and Analogs

Compd	R^1	R^2	R^3	R^4	IC_{50} (μM)[a] MDR-reversal	IC_{50} (μM)[b] Cytotoxicity	MDR : Cytotoxicity Window[d]
verapamil[c]	---	---	---	---	3.1	53.0	17.1
hapalosin	*i*-Pr	Me	*n*-C_7H_{15}	OH	4.8	14.8	3.0
1	"	"	"	OMOM	6.1	34.9	5.7
2	Me	"	"	OH	6.2	27.1	4.3
3	"	"	"	OMOM	5.9	19.3	3.2
4	H	"	"	OH	5.8	22.5	3.8
5	"	"	"	OMOM	2.3	15.1	6.5
6	*i*-Pr	H	"	OH	6.2	24.0	3.8
7	"	"	"	OMOM	6.0	34.4	5.7
8	"	Me	Me	OH	15.0	>100	>9.0
9	"	"	"	OMOM	7.5	>75	>10
10	"	"	H	OH	21.9	>100	>7.5
11	"	"	"	OMOM	10.4	>100	>15.9
12	"	"	*n*-C_7H_{15}	H	6.0	33.7	5.6
13	"	"	"	α-OH	5.7	42.2	7.4
14	"	"	"	α-OMOM	5.5	41.5	7.5
15	*gem*-di-Me	"	"	OH	4.3	28.4	6.6
16	"	"	"	OMOM	5.6	20.0	3.5
17	H	"	Me	OH	>100	>100	---
18	"	"	"	OMOM	42.7	>100	>2.3
19	"	"	*n*-C_4H_9	OH	18.1	>80	>4.4
20	"	"	"	OMOM	11.7	>100	>8.5
21	*i*-Pr	Me (aldol diast.)	*n*-C_7H_{15} (aldol diast.)	OH	5.8	31.3	5.4
22	"	"	"	OMOM	5.4	33.7	6.2
23	---	---	---	---	14.9	>86	5.7
24	---	---	---	---	17.1	>89	5.2
25	---	---	---	---	>100	>100	---
26	---	---	---	---	16.0	>83	>5.2

[a] average IC_{50} (n = 2 or 3) in the presence of 10 nM vincristine.

[b] average IC_{50} (n = 2 or 3) in the absence of vincristine.

[c] average IC_{50} (n = 20), used as a positive control in all assays.

[d] window = IC_{50}[cytotoxicity]/IC_{50}[MDR-reversal].

Discussion: In our cell-based assay, synthetic hapalosin was somewhat less active than verapamil and showed only a threefold concentration difference between cytotoxicity and MDR-reversing activity (Table 1). Given the modular approach to the synthesis of hapalosin via the fragments **A**, **B**, and **C**, we felt it would be very simple to vary the peripheral ring substituents in an effort to differentiate the MDR-modulating activity from cytotoxicity. Accordingly, we choose to modify R^1 - R^4 in a systematic fashion as these types of changes had not been disclosed in any of the previous literature references.[13]

Hapalosin exists as an ca. 2:1 mixture of tertiary amide rotamers[4a] in solution, and it has been suggested that the major s-*cis* amide is the biologically active one.[5c,f,g] Somewhat surprisingly, changing the isopropyl group (R^1) of hapalosin to a methyl (**2**) did not significantly alter the activity even though essentially only one rotamer was observed by ^{1}H NMR.[14] Changing R^1 to hydrogen (**4**) also gave a slight loss of activity (2.5:1 rotamer ratio); however, our most active analog (**5**, 2 x more active than hapalosin) was found when the hydroxy group was protected as the MOM-ether (2:1 rotamer ratio). A small decrease in activity was observed when the methyl substituent (R^2) was replaced with hydrogen (**6**), and shortening of the heptyl sidechain (R^3) to methyl (**8**) or a hydrogen (**10**) resulted in progressively larger losses of activity. Surprisingly, when the 2°-hydroxyl group (R^4) was either removed (**12**) or the absolute configuration at that center was changed (**13** or **14**, essentially one rotamer for the latter), these analogs showed only a slight loss in their MDR-reversing abilities. Increasing the bulk of R^1 to a *gem*-dimethyl center did not significantly alter the amide rotamer ratio nor the activity of the analogs (**15/16**), and changing the relative stereochemistry between R^2 and R^3 (**21/22**) did not provide any improvement in the MDR-reversing activity. The four fragments (**23-26**) were considerably less cytotoxic than hapalosin, but also less active as well.

Given the improved MDR-reversal of **5** (with no increase in cytotoxicity) and the decreased cytotoxicity of **9** (without a significant loss of MDR activity), we wanted to combine those two structural modifications to see if the effects would be additive. This lead to the preparation of analogs **17/18**, which were not very cytotoxic and also not very active for MDR-reversal. These results may be partly rationalized by invoking a decrease in cellular permeability for these less lipophilic analogs. Adding back three carbons to the R^3 position (**20/21**) restored some MDR-reversing ability while still providing an improved effectiveness window relative to cytotoxicity, supporting this hypothesis.

Summary: The main goal of this project was to determine if MDR-reversing activity and general cytotoxicity could be dissociated by substituent changes on the macrocyclic scaffold of hapalosin. This turned out to be possible to a limited extent, as many analogs showed a larger window between these activities than did hapalosin. Unfortunately, most analogs were also less active or only comparable in activity to the parent compound as MDR-reversing agents. The overall contributions of various factors to the macrocycle conformation (amide rotamers, internal H-bonding of the 2°-hydroxy group, ring flexibility) as well as the pattern of substituents presented to P-gp appear to be closely interconnected and not readily discernible. Our initial study of variations on hapalosin's substituents R^1 - R^4 have indicated that a simple differentiation of desired activity and cytotoxicity in a potent MDR-reversing analog is probably not possible, and that more drastic modifications to the molecule will be needed to provide a clinically useful compound.

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