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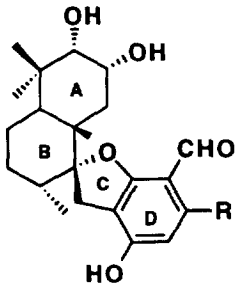
FURTHER STRUCTURE-ACTIVITY-RELATIONSHIP STUDIES ON A/C/D-RING ANALOGS OF COMPLEMENT INHIBITOR K-76

Ranjan P. Srivastava,† Xiaoyan Zhu,† Larry A. Walker†,§ and Robert D. Sindelar†,‡,*

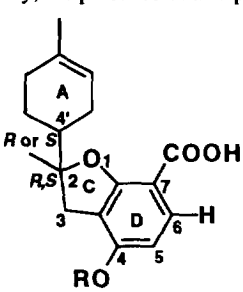
†*Research Institute of Pharmaceutical Sciences,* §*Department of Pharmacology and* ‡*Department of Medicinal Chemistry, School of Pharmacy, The University of Mississippi, University, Mississippi 38677*

Abstract: A series of new A/C/D-ring analogs (**4a-f, 15**) of fungal metabolite K-76 (**1a**) have been synthesized and evaluated for human complement inhibitory potency. The *in vitro* assay results of human complement-mediated hemolysis of A/C/D-ring analogs indicate that the carboxylic acid functionality at C-6 is more important than C-7. The presence of aldehydic group and the terpenoid diol in these analogs contribute little towards human complement inhibition. The role of the phenolic hydroxyl is critical as benzofurans **4a** and **4b** exhibit human complement inhibition similar to the natural product.

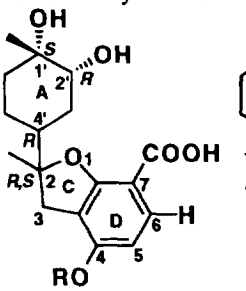
An interest in the development of complement inhibitors has emerged from a greater understanding of the involvement of the complement system in several disease process.[1-3] The discovery of K-76 (**1a**), a natural product of fungal origin, and its oxidized derivative **1b** (R = COOH; K-76COOH), as selective inhibitors of the complement cascade[4] prompted us to synthesize a potential new class of complement inhibitors, using the concept of "structure pruning" on the molecular framework of **1a,b**. In a continuation of our program[5,6] to design A/C/D-ring analogs of **1a,b**, retaining the desired complement inhibiting potency, we have synthesized several compounds of prototypes **2** and **3**. The *in vitro* assay results[5,6] of their inhibition of classical complement-mediated hemolysis have indicated that the (4'*S*)-diastereomer may be more potent than the (4'*R*)-isomer (Table 1). Additionally, the presence of a terpenoid diol may not be essential for complement inhibition.



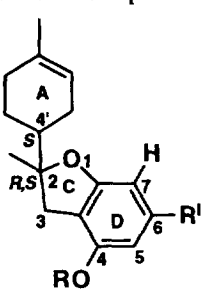
1a: R = CHO; K-76
1b: R = COOH; K-76COOH
1c: R = COONa; K-76COONa



2a: 4'*R*, R = H
2b: 4'*S*, R = H
2c: 4'*R*, R = Me
2d: 4'*S*, R = Me



3a: R = H
3b: R = Me



4a: R = H, R' = CHO
4b: R = H, R' = COOH
4c: R = Me, R' = CHO
4d: R = Ph, R' = CHO
4e: R = Me, R' = COOH
4f: R = Ph, R' = COOH

In this series of analogs belonging to prototypes **2** and **3**, the carboxylic acid functionality was attached to C-7 rather than C-6, thus not corresponding to the carboxyl group position as found in the lead compound **1b**. Additionally, previous studies did not explore the role of the phenolic hydroxyl group in **1a**. Therefore, herein,

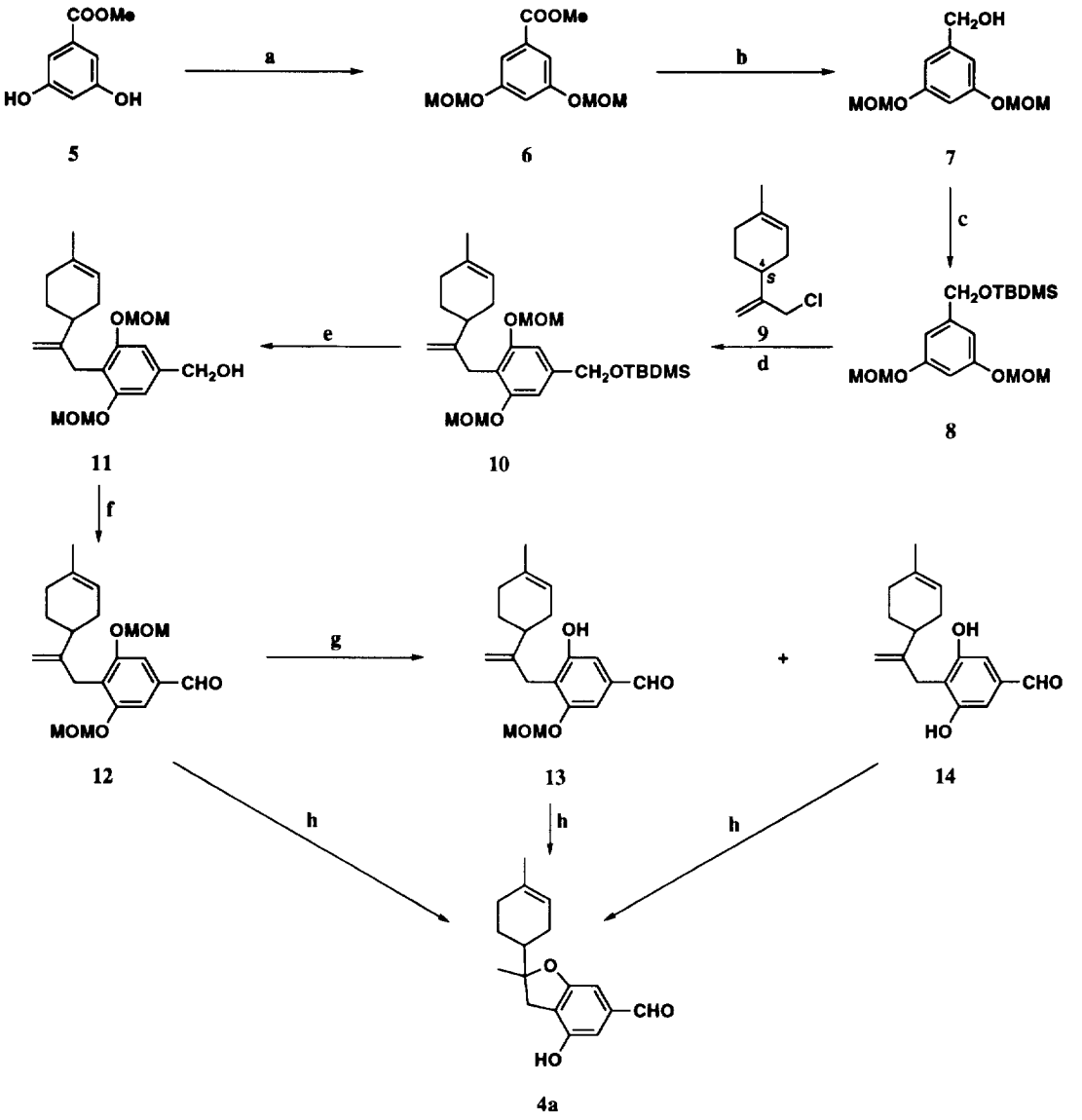
we report the design and synthesis of new A/C/D-ring analogs of **1a,b** (**4a-f, 15**) which not only contain the carboxylic acid group at the appropriate position (C-6), but also examines variation at C-4. This structure-activity-relationship study helps elucidate the essential pharmacophore of this important natural product.

Our synthetic strategy utilizes methyl 3,5-dihydroxybenzoate (**5**) as the starting material and has the following salient features: (a) the preparation of 3,5-bis(methoxymethoxy)benzyl alcohol *tert*-butyl dimethylsilyl ether (**8**) to introduce ring D of the proposed analogs; (b) the coupling of **8** with (4*S*)-(–)-limonene chloride (**9**; the A-ring source); (c) the deprotection of *tert*-butyldimethylsilyl (TBDMS) and methoxymethyl (MOM) protected hydroxyls; and (d) the ring closure of 4-(substituted propen-3'-yl)benzaldehyde derivatives (**13,14**) to generate the C-ring.

The silyl ether **8** was synthesized from ester **5** according to the method reported earlier.[7,8] The protection of resorcinol **5** as MOM ether followed by LAH reduction gave the benzyl alcohol **7** which subsequently was reacted with TBDMSCl to generate the silyl ether intermediate **8**. The coupling of **8** with (4*S*)-(–)-limonene chloride[9] (**9**) was successfully accomplished by the treatment of an aryl cuprate reagent (formed by the reaction of TMEDA–*n*-BuLi and CuI with **8** in dry tetrahydrofuran) with **9**, yielding 82% of the key intermediate **10** as a colorless oil (Scheme 1). Reaction of **10** with tetrabutylammonium fluoride gave the benzyl alcohol **11** which after PCC oxidation furnished the benzaldehyde **12** in 90% yield. Compound **12** was then subjected to mild acidic hydrolysis (3 N HCl/2-propanol) in order to deprotect the MOM protected phenols. After chromatographic separation of the reaction mixture, three fractions were obtained which were characterized as recovered **12** (7%) and as mono- and dihydroxybenzaldehyde derivatives (**13,14**) in 21% and 52% yields respectively. Compounds **13** and **14** were separately treated with Amberlyst 15 in dichloromethane which furnished exclusively benzofuran **4a** in good yield. Based on this observation, compound **12** was directly subjected to treatment with Amberlyst 15 in dichloromethane. In addition to the desired product **4a**, a complex mixture of **12**, **13**, **14** and other difficult to isolate unidentified products was obtained which revealed this reaction to be problematic. Therefore, the 3 N HCl/2-propanol-mediated deprotection described above followed by cyclization in the presence of Amberlyst 15 was preferred in this synthetic scheme. The resulting benzofuran **4a** was treated with silver nitrate and aqueous potassium hydroxide in ethanol to yield the corresponding target acid **4b** as a white solid (63%). The reaction of **4a** with iodomethane or triphenylbismuith diacetate[10] successfully gave the methoxy or phenoxy derivative **4c** or **4d** in 95% and 75% yields respectively (Scheme 2). Compounds **4c** and **4d** were then easily converted to their corresponding acids **4e** and **4f** following the same procedure as described earlier. Compound **4e** could be dihydroxylated by treatment with osmium tetraoxide and trimethylamine *N*-oxide in *tert*-butanol to furnish an additional analog **15** in 68% yield.

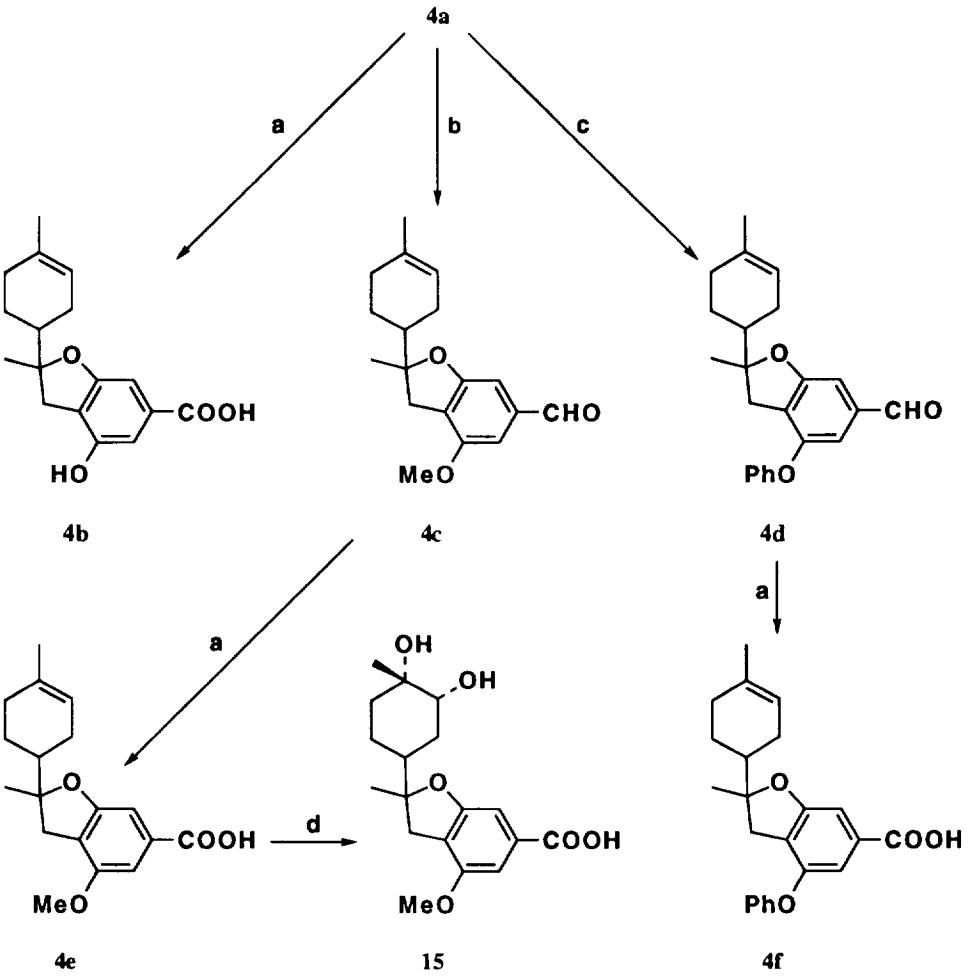
The target compounds described above were assayed for their ability to inhibit complement activation. For the estimation of inhibition of the classical complement pathway, the method of Weisman et al.[11] was adapted to a 96-well microplate format to reduce sample size, conserve reagents, and increase throughput. Lyophilized human complement (Diamedix, Cat. # 789-006, Miami, Fl) was reconstituted with 3 mL cold phosphate buffered saline (PBS), then diluted in phosphate buffered saline 1:400 for use in the assay. Test compounds were dissolved in DMSO at a concentration of 12 mg/mL, then diluted with PBS to 1.2, 0.6 and 0.3 mg/mL. The appropriate concentrations of test compounds (50 μL) were added to 96-well round bottom plates (Falcon 3077), followed by 10 μL of the diluted complement. After equilibration for 15 min, 150 μL of sensitized sheep erythrocytes (SRBCs) (Diamedix, Cat. # 789-001, Miami, FL) were added to the wells and incubated at 37 °C for 1 h. After incubation, plates were centrifuged at low speed to pellet the intact SRBCs. One

Scheme 1[a]



[a] Reagents: (a) MOMCl, NaH, DMF; (b) $LiAlH_4$, THF; (c) TBDMSCl, Imidazole, CH_2Cl_2; (d) *n*-BuLi, TMEDA, CuI; (e) $(Bu)_4NF$, THF; (f) PCC, CH_2Cl_2; (g) 3 N HCl, 2-PrOH; (h) Amberlyst 15, CH_2Cl_2.

Scheme 2[a]



[a] Reagents: (a) $AgNO_3$, KOH, ETOH, H_2O; (b) CH_3I, Ag_2O, $CHCl_3$; (c) $Ph_3Bi(OAc)_2$, Cu, CH_2Cl_2; (d) OsO_4, $(Me)_3N(O).2H_2O$, *t*-BuOH.

hundred μL of the supernatant was transferred to flat bottom plates (Falcon 3075), and read on a Biotek EL312 plate reader (405 nm with 630 nm background subtraction). Each compound was run in triplicate at each concentration. Appropriate controls were included: vehicle (DMSO), positive control complement inhibitor (**1c**, supplied by Otsuka Pharmaceuticals), maximum lysis controls (0.2% SDS). All test compounds were also incubated with SRBCs in the absence of complement to determine their lytic activity. Inhibition of complement was evaluated by comparison of test compounds with vehicle controls. Response data for complement inhibition were calculated according to the formula:

$$\% \text{ Hemolysis} = (\mathbf{Abs}_{test} - \mathbf{Abs}_{bkgd}) / (\mathbf{Abs}_{veh} - \mathbf{Abs}_{bkgd}) \times 100$$

where Abs_{test}, Abs_{veh}, and Abs_{bkgd} are the corrected absorbance readings (405-630 nm) for test wells (complement plus inhibitor), complement wells with vehicle, and no complement wells (no hemolysis), respectively. Lytic activity of the samples were calculated by :

$$\% \text{ Hemolysis} = (Abs_{lyt} - Abs_{bkgd}) / (Abs_{veh} - Abs_{bkgd}) \times 100$$

where Abs_{lyt} is the reading for wells with test compound in the absence of complement, and other terms are as described above. DMSO did not affect complement activity at final assay concentrations of 0.5 to 2%. Concentration-response data were plotted and IC_{50} (50% inhibitory concentration) or EC_{50} (concentration for 50% hemolysis by test compound) were derived graphically.

Table 1. The Inhibition of Human Complement Activation and Intrinsic Lytic Activity of Target Compounds.

Compound	IC_{50} (μM)[a] Complement Inhibition	EC_{50} (μM)[b] Lysis
1c	680	>680
2a	2100[c]	d
2b	2400[c]	d
2c	680[c]	d
2d	380[c]	d
3b	1600[e]	d
4a	515	<280
4b	730	>1040
4c	>1049	>1049
4d	>862	>862
4e	>1000	660
4f	>824	<200
15	>892	>892

[a] The concentration of compound required to inhibit complement induced hemolysis by 50% comparable to vehicle (DMSO). Values reported are interpolated from concentration/inhibition plots of mean values (n = 3 at each concentration).

[b] The concentration of compound required to cause 50% hemolysis in the absence of complement. Values are interpolated from concentration/lysis plots of mean values (n = 3 at each concentration).

[c] Reported in reference 5, incuded for comparison.

[d] Not bioassayed.

[e] Reported in reference 6, included for comparison.

The *in vitro* inhibition of classical human complement-mediated hemolysis indicates that benzofurans **4a** and **4b** are active in the same range of concentrations as the natural product (Table 1). These results together with

our previous findings,[5,6] furnish a consistent structure-activity relationship of A/C/D-ring analogs, suggesting that the presence of the aldehydic group in the D-ring may not be an essential requirement for complement inhibition. However, the potency of the A/C/D-ring analogs is improved by a carboxylic acid functionality at C-6 rather than at C-7. A very significant finding is that, unlike A/C/D-ring analogs with the carboxyl function in position 7,[5] the role of phenolic hydroxyl is critical for potency of the analogs with the carboxyl function at position 6 (**4a**, **4b**). Little activity was observed in the methoxy derivative **4e**, differing significantly from the analogs in which the COOH group was at C-7 position.[5] Also, both phenoxy derivatives **4d** and **4f** were inactive.

Changing the lypophilicity in the D- or A-ring of the closely related A/C/D-ring analogs affects the intrinsic lytic activity significantly as observed from the comparision (more lytic/less lytic) of compounds **4a/4b**; **4a/4c**; **4a/4d**; **4e/4b**; **4f/4b**; **4e/4c**; **4f/4d**; **4f/4e** and **4e/15**.

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