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Statin-derived 1,3-oxazinan-2-ones as submicromolar inhibitors of LFA-1/ICAM-1 interaction: stabilization of the metabolically labile vanillyl side chain

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Abstract—Modification of the vanillyl substituent on a potent, semisynthetic lymphocyte function-associated antigen (LFA)-1/intercellular adhesion molecule (ICAM)-1 binding inhibitor of the statin family resulted in metabolically more stable analogues that displayed submicromolar inhibitory activity in vitro and considerable anti-inflammatory activity in vivo. The benzodioxole derivative **2b** emerged with the best overall profile.

Recent studies established that 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) display direct anti-inflammatory effects that are presumably not mediated by their known cholesterol-lowering properties.[1] High throughput screening of large chemical libraries has identified lovastatin (mevinolin, **1**, Fig. 1) as an extracellular inhibitor of LFA-1/ICAM-1 adhesion and T-cell co-stimulation.[2] As documented by NMR spectroscopy and crystallography, lovastatin binds to an allosteric site in the protein domain ('L-site') of LFA-1 and apparently stabilizes a low-affinity conformation of this receptor.[3a,b] Since interactions of cell adhesion molecules (CAM) play central roles in mediating immune and inflammatory responses,[4a–d] the inhibition of the interaction of LFA-1 with its ligand ICAM-1 potentially represents a new approach to immunosuppression and anti-inflammatory treatment. As clinical data show that a humanized anti-LFA-1 mAb is efficacious in patients suffering from moderate to severe plaque psoriasis,[5] there has been an increasing effort to discover small molecule inhibitors in recent years.[6]

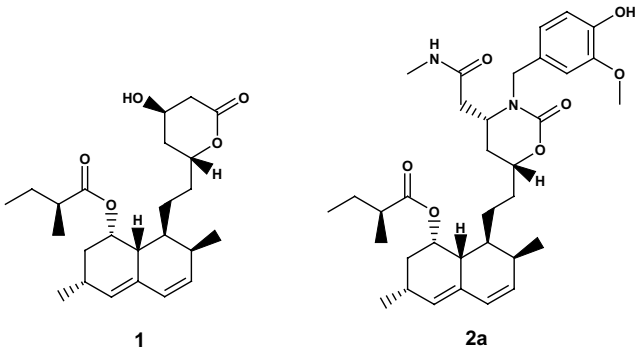

Figure 1. Mevinolin and the lead compound, **2a**.

Guided by the crystal structure of the complex between the LFA-1 binding domain and lovastatin, a series of statin-based compounds had been synthesized and screened in a cell-free ELISA-type LFA-1/ICAM-1 binding assay and also in a cellular human T-cell/ICAM-1 adhesion assay.[7–9] The major goal was to discover novel, lovastatin-derived, semisynthetic ligands with submicromolar IC_{50} ($IC_{50} = 3.78\ \mu M$ for lovastatin in the cell-free assay).[9] From this set of compounds, **2a** (a 1,3-oxazin-2-one bearing an *N*-vanillyl side chain, Fig. 1) emerged as a promising lead structure ($IC_{50} = 0.05\ \mu M$).[8] Its favorable binding profile,

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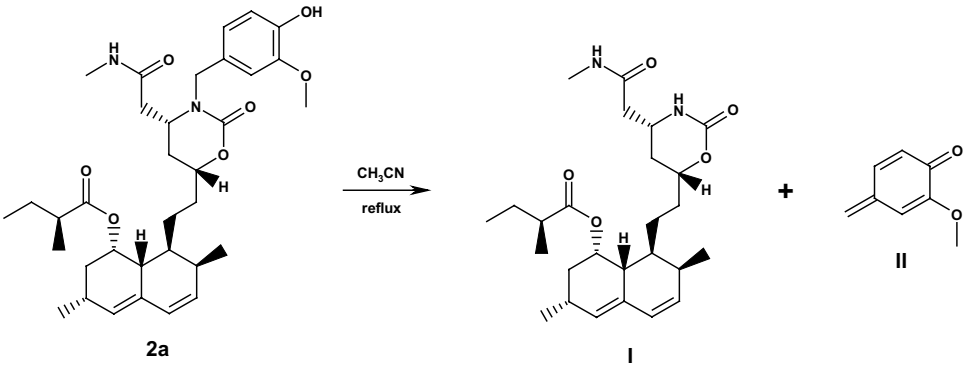
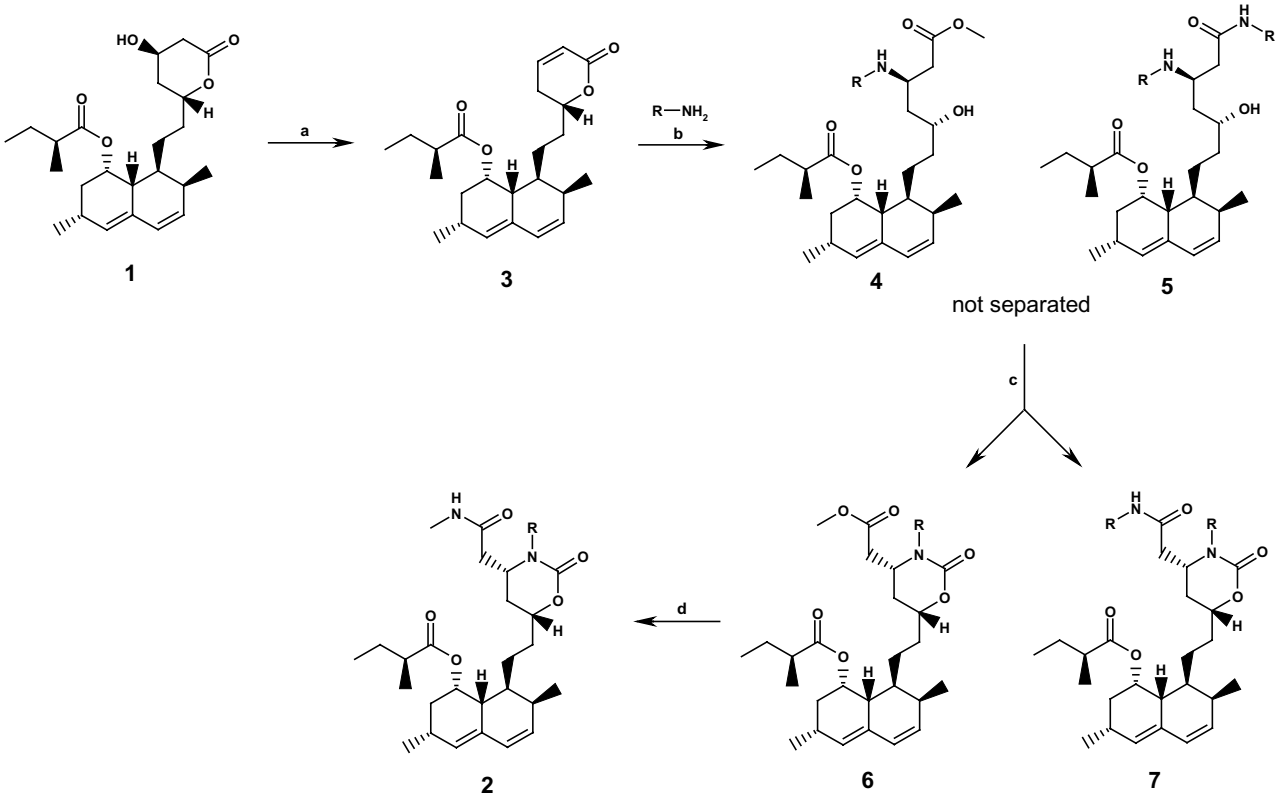
however, appears to be compromised by drug-unlike structural features. A major matter of concern was the *N*-vanillyl side chain that may undergo phase II metabolism (phenol glucuronidation) and exhibits a chemically labile, benzyl-type C–N bond. It was shown in our group that **2a** degrades upon heating to fragment **I**, probably via formation of a highly reactive *p*-quinone methide (fragment **II**, Scheme 1). We thought that the chemical instability and the metabolic vulnerability of **2a** could be addressed by replacing the free hydroxyl group or modifying the carbon skeleton of the vanillyl side chain. In this paper, we present the synthesis, biological activity, and microsomal stability of such less vulnerable **2a**-type compounds.

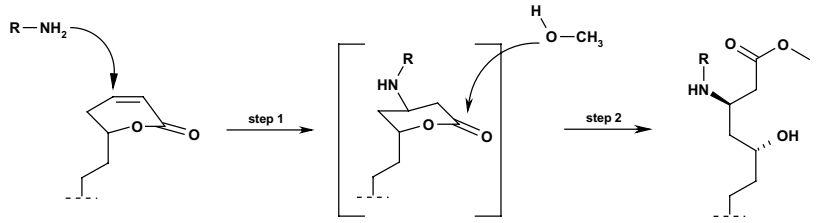
Compound **2a** and its analogues were all synthesized following the strategy outlined below (Scheme 2). A detailed experimental procedure and analytical characterization for **2a** has been reported in patent literature.[7] Mevinolin (**1**) was mesylated and the mesylate eliminated to furnish the α,β-unsaturated lactone **3**. Upon addition of the appropriate primary amine in a methanolic solution, a two-step process took place whereby the nucleophilic attack of the amino nitrogen is accompanied by transesterification of the lactone to give β-amino-δ-hydroxy methyl ester **4**. Interestingly, the ratio of diastereomers with respect to the newly formed chiral center was determined to be *R*:*S* ~ 9:1, independently of which amine was employed. Obviously, the



Scheme 1. Degradation of **2a**.



Scheme 2. Reagents and conditions: (a) CH_3SO_2Cl, DMAP, CH_3CN, rt, 90%; (b) MeOH, rt, 40–50%; (c) CDI, toluene/CH_3CN, 90%; (d) CH_3NH_2, MeOH, rt, 85%.



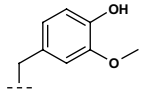
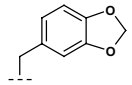
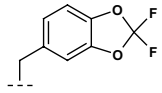
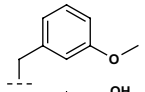
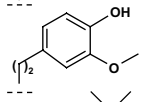
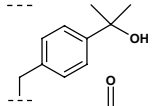
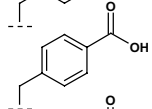
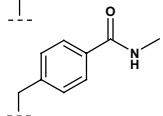
Scheme 3. Suggested mechanism of ring opening.

attack of the amine occurs preferably from the less hindered side (see Scheme 3). It can be therefore concluded that the chiral center adjacent to the lactone sp^3 oxygen induces a remarkably stereoselective attack on the C–C double bond. While this selectivity seems plausible on the strained ring, we reasoned that it would not occur to that extent on an open-chain intermediate. This led to the assumption that Michael addition takes place *before* alcoholysis of the lactone. Ester **4** was obtained in 40–50% yield. Excess amine reagent usually enhanced the conversion rate, but with increasing time, aminolysis of the methyl ester led to by-product **5**.

Without separating **4** and **5**, the mixture of the two compounds was treated by carbonyl diimidazole (CDI) to give the cyclic carbamates (1,3-oxazin-2-ones) **6** and **7**. At this stage, chromatographic separation yielded the desired compound **6**. This purification step also enabled the enrichment of the major diastereomer from 9:1 to at least 95:5. Finally, purified esters **6** underwent aminolysis with $MeNH_2$ to afford the test compounds **2**.[10]

N-Methyl amides **2a**–**h** were submitted to two in vitro LFA-1 binding assays (inhibition of the interaction of recombinant ICAM-1 with purified, immobilized LFA-1, as previously described;[3] inhibition of the adhesion of HUT78 cells to immobilized ICAM-1 protein, as previously described),[2] and to a murine model of allergic contact dermatitis (mACD) for testing anti-inflammatory activities after single oral administration[11,12] (Table 1). Compounds **2a**–**h** were also tested for microsomal stability,[13] whereas **6a**–**h** were not considered, or

Table 1. Pharmacokinetic and biological data for **2a**–**h** and **6a**–**h**

Entry	R	Clearance (μL/min/mg)		IC_{50} (μM)[a] (cell-free assay)	IC_{50} (μM)[a] (adhesion assay)	mACD (% inhibition of inflammatory swelling)[b]	
		Human liver microsomes	Rat liver microsomes			1.0 mg/kg	0.1 mg/kg
2a		>500	>500	0.05	0.25	$47 \pm 2.1^{***}$	$39 \pm 2.6^{***}$
6a				0.05	0.12	$46 \pm 6.8^{**}$	168.6^{ns}
2b		383	100	0.17	6.42	$29 \pm 5.9^{**}$	$28 \pm 6.0^{**}$
6b				1.00	2.59	$42 \pm 4.9^{***}$	$32 \pm 5.9^{***}$
2c		>500	207	1.15	5.59	19 ± 9.3^{ns}	n.d.
6c				>30	>30	-3 ± 7.7^{ns}	n.d.
2d		55	123	0.27	6.50	21 ± 7.4^{ns}	n.d.
6d				0.68	4.50	$23 \pm 5.9^{*}$	n.d.
2e		>500	>500	0.80	9.50	$43 \pm 4.8^{***}$	$29 \pm 7.8^{*}$
6e				0.64	20.5	$30 \pm 4.1^{***}$	9 ± 9.9^{ns}
2f		>500	>500	1.50	n.d.	26 ± 11.7^{ns}	9 ± 7.3^{ns}
6f				3.00	7.69	33 ± 6.3^{ns}	12 ± 9.4^{ns}
2g		205	214	0.87	>30	21 ± 8.6^{ns}	n.d.
2h		>500	>500	0.62	>30	$26 \pm 6.4^{**}$	$32 \pm 6.4^{**}$
6h				1.30	>30	8 ± 9.3^{ns}	n.d.

$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus vehicle-treated controls; ns: not statistically different from controls; n.d.: not determined.

[a] Values are means of three independent experiments.

[b] Mean ± SEM, eight animals per group; statistically significant inhibitions by >25% considered to be biologically relevant.

regarded as potential drug candidates, since our own experience with similar compounds has taught us that methyl esters (unless sterically hindered) are readily cleaved by esterases in most organs and blood, particularly in rodents. Metabolic stability of such compounds is usually so low that the obtained clearance values would most likely not correlate with biological results.

Compounds **2e** and **6b** showed pronounced anti-inflammatory activity in the mACD model (>40% inhibition at 1.0 mg/kg; ~30% at 0.1 mg/kg). In the in vitro assays, however, both compounds were markedly less active than **2a** (cell-free assay: 16- and 20-fold, cellular: 38- and 10-fold, respectively). Interestingly, **2e** is the only analogue, which features a phenolic group and a stable phenethyl side chain. Acetal masking of the phenol, as shown in benzodioxole **2b**, led to retention of activity in the cell-free LFA-1 assay ($IC_{50} = 0.17\,\mu M$), compromising activity in the cellular assay ($IC_{50} = 6.42\,\mu M$) and in vivo efficacy (~30% inhibition at both doses). All the other test compounds (**2c**–**h**) displayed a mediocre in vitro profile (cell-free assay: IC_{50} values >0.2 μM, cellular assay: IC_{50} values >5 μM). We therefore assumed that the pronounced in vivo anti-inflammatory activity was highly dependent on the presence of the phenolic group (as in compounds **2a** and **2e**). Indeed, an attenuation of in vivo activity could be observed when the phenol was masked, but could be presumably 'deprotected' in vivo to restore the active principle (as in acetals **2b** and **6b**). In the case of difluorobenzodioxoles **2c** and **6c**, no such conversion was expected,[14] and these compounds showed strongly reduced in vivo activity, like all other compounds lacking a free or masked phenolic hydroxyl group. Compounds with other functions on the benzylic side chain, such as COOH (**2g**) or $CONH_2$ (**6h** and **2h**), lacked activity in the LFA-1 dependent cellular adhesion assay (IC_{50} values >30 μM) whereas submicromolar activity in the cell-free binding assay was retained. In general, it was observed that all derivatives of **2a** were more active in the cell-free binding assay than in the cell-based adhesion assay (usually by a factor of >10), highlighting a common issue with the substance class that had previously been concealed by the good overall profile of **2a**. A comparison of the precursor compounds (methyl esters **6a**–**h**) with the methyl amides (**2a**–**h**) implies that the majority of amides show a better profile in the cell-free LFA-1/ICAM-1 binding assay than the corresponding methyl esters; the cellular assay and the in vivo data do not show a preference for either substance class, respectively.

Importantly, the masking or elimination of the phenolic OH resulted in a pronounced improvement of the poor metabolic stability observed for **2a** (intrinsic clearance CL_i >500 μL/min/mg in both human and rat liver microsomes). In comparison to **2a**, compounds **2b** and **2c** (hydroxyl group masked) show markedly decreased values when exposed to rat liver microsomes (100 and 207 μL/min/mg, respectively). The homovanillyl derivative **2e** exhibits poor metabolic stability (human and rat: >500 μL/min/mg), being indicative for the liability of the vanillyl moiety. Compound **2d** (which is devoid of the hydroxyl group but still bears the methoxy substituent) has a drastically improved metabolic profile for both species (human: 55 μL/min/mg, rat: 123 μL/min/mg).

We have shown that the adverse metabolic stability profile of lead compound **2a** can be addressed by minor modifications of its labile vanillyl substituent, but at the expense of either in vitro or in vivo activity (or both). Thus, elimination of the phenolic OH or its masking as an acetal led to improved microsomal stability when exposed to liver microsomes while sacrificing only little of its cell-free activity but much of its cellular anti-adhesive and in vivo activity. The discrepancy between the in vitro and in vivo profiles of some compounds may suggest species specificity of the ligands, as both the cell-free and the cellular LFA-1 binding assays are based on human proteins. It is also conceivable that the potent *N*-vanillyl derivatives exhibit part of their anti-inflammatory activity in mice via a hitherto unknown, LFA-1 independent mechanism. Among the derivatives synthesized, **2b** emerged with the overall best activity and stability profile: It shows IC_{50} values of 0.17 and 6.42 μM in the cell-free and cellular assay, respectively, significantly inhibits inflammation in the mACD animal model by 28% even at the low dose (0.1 mg/kg), and shows in vitro clearance values of 383 μL/min/mg (human microsomes) and 100 μL/min/mg (rat microsomes). None of the other amides with improved microsomal stability are significantly active in the mACD model, not even at the higher dose (1.0 mg/kg). Compound **2b**, as the only compound fulfilling our criteria, was selected as a novel lead for further investigations.

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10. All tested compounds showed a chemical purity of >95%, as determined by ^1H NMR and HPLC. Further charac-

terization was performed with ^{13}C NMR, two-dimensional NMR analysis, and mass spectrometry.

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12. Groups of eight oxazolone-sensitized mice were challenged with 10 μL of 2.0% oxazolone on the inner surface of the right ears to elicitate ACD. The unchallenged left ears served as normal controls and dermatitis was evaluated from the individual differences in auricular weights, which were taken as a measure of increase in inflammatory swelling 24 h after the challenge. The test groups were orally treated with the test compounds 2 h after challenge. The data of the test groups and vehicle-treated control groups were statistically analyzed by ANOVA followed by Dunnet T-test (normal distribution of data) or by H and U-test, respectively.
13. For determination of microsomal stability, pooled hepatic microsomes were obtained from Xenotech LLC (Kansas City, KS) or In Vitro Technologies (Baltimore, MD). Singular microsomal incubations were carried out at a test concentration of 1 μM and a protein concentration of 0.15 mg/mL in a reaction mix containing 100 mM phosphate buffer, 5 mM $MgCl_2$, 1 mM EDTA, 0.35 mg/mL albumin, and 9 μg/mL almethacin at pH 7.4 at 37 °C. To initiate the incubations, an aqueous cofactor solution was added to each tube giving a final concentration of 4 mM UDPA and 1 mM NADPH. At 5 and 20 min, respectively, incubations were terminated by adding and mixing with ACN containing 0.02% FA (v/v). The incubations were put in a freezer at −20 °C for 2 h and were subsequently centrifuged at 4000 rpm for 20 min. Aliquots of the supernatant (60 μL) were transferred into a 96-well plate. The plate was stored at −20 °C until analyzed.
14. The difluorobenzodioxole group has been shown to be extremely stable toward hydrolysis across a broad pH range: Schlosser, M.; Gorecka, J.; Castagnetti, E. *Eur. J. Org. Chem.* **2003**, 452.