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Identification of *N*-acyl 4-(3-pyridonyl)phenylalanine derivatives and their orally active prodrug esters as dual acting α4β1 and α4β7 receptor antagonists

Jefferson W. Tilley [a,*], Achyutharao Sidduri [a], Jianping Lou [a], Gerry Kaplan [a], Nadine Tare [b], Gary Cavallo [b], Karl Frank [c], Anjula Pamidimukkala [c], Duk Soon Choi [d], Louise Gerber [d], Aruna Railkar [d], Louis Renzetti [b]

[a] Discovery Chemistry, Pharmaceutical Research and Early Drug Development, Hoffmann-La Roche Inc., 340 Kingsland Street, Nutley, NJ 07110, USA
[b] Discovery Inflammation and Respiratory Diseases, Pharmaceutical Research and Early Drug Development, Hoffmann-La Roche Inc., 340 Kingsland Street, Nutley, NJ 07110, USA
[c] Drug Metabolism and Pharmacokinetics, Pharmaceutical Research and Early Drug Development, Hoffmann-La Roche Inc., 340 Kingsland Street, Nutley, NJ 07110, USA
[d] PARD, Pharmaceutical Research and Early Drug Development, Hoffmann-La Roche Inc., 340 Kingsland Street, Nutley, NJ 07110, USA

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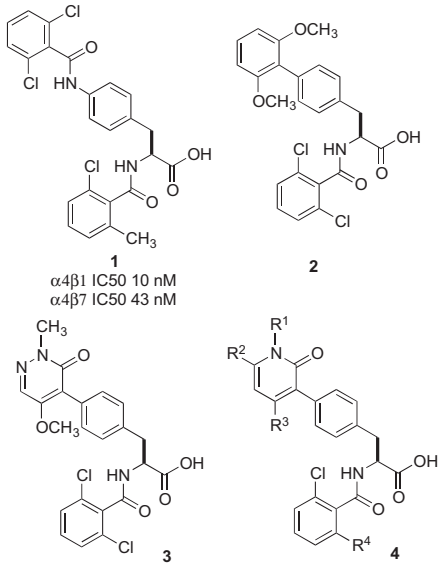
ABSTRACT

From a series of *N*-acyl 4-(3-pyridonyl)phenylalanine derivatives of **4**, the trifluoromethyl derivative **28** was identified as a potent, dual acting alpha4 integrin antagonist with activity in primate models of allergic asthma. Investigation of a series of prodrug esters led to the discovery of the morpholinopropyl derivative **48** that demonstrated good intestinal fluid stability, solubility and permeability. Compound **48** gave high blood levels of **28** when dosed orally in cynomolgus monkeys. Surprisingly, hydrolysis of **48** was rapid in liver microsomes from the pharmacological species, mouse, rat and monkey, but slow in dog and human; in vivo studies also indicated there was prolonged exposure to unchanged prodrug in dogs.

Alpha4 integrins are expressed on a variety of leukocytes, including B-cells, T-cells, basophils and eosinophils, and are involved in the recruitment, activation and survival of these cell types. Data supporting a role for alpha4 integrins in a number of inflammatory diseases, including multiple sclerosis, inflammatory bowel diseases, asthma, rheumatoid arthritis, and atherosclerosis, have emerged and are summarized in recent reviews.[1] A humanized mouse anti-alpha4 antibody, Natalizumab, has been approved for the treatment of multiple sclerosis[2] and Crohn's disease.[3] More recently a small molecule alpha4 integrin antagonist was shown to be effective for the treatment of MS,[4] fully validating alpha4 integrins as targets for human disease and increasing interest in the discovery of small molecules with superior activities.

We previously reported that the potent, dual-acting antagonist of α4β1 and α4β7, **1,** is effective for the treatment of asthma in man[5] and sought additional compounds that might have lower clearance and greater selectivity for α4β7 to further assess their potential as alpha4 integrin antagonists. Inspired by reports of the alpha4 integrin antagonist activity of members of the biphenylalanine class such as **2**,[6] as well as the interesting, selective integrin antagonist activity of members of the pyridizinone family represented by **3**,[7] we report here on a new series of potent alpha4 integrin antagonists, *N*-acyl 4-(3-pyridonyl)-phenylalanine derivatives **4** and their orally active prodrug esters.

The compounds reported in Table 1 were typically prepared by a palladium-catalyzed coupling reaction of the 4-iodophenylalanine derivative **17** with an in situ generated organozinc intermediate derived from the appropriate 4,6-disubstituted 3-iodo-pyridone intermediate such as **11** and **16** (Scheme 3).



1
α4β1 IC50 10 nM
α4β7 IC50 43 nM

2

3

4

* Corresponding author.
E-mail address: tilleyjk@optonline.net (J.W. Tilley).

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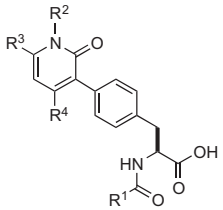
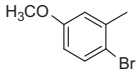
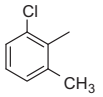
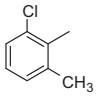
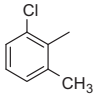
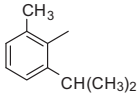
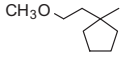
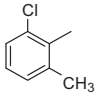
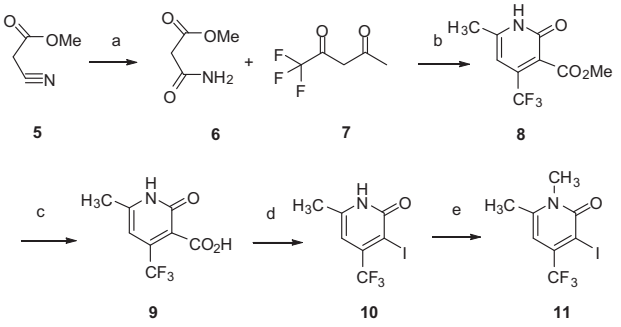
Table 1
VCAM/VLA-4 binding inhibition of *N*-acyl 4-(3-pyridonyl)-L-phenylalanine derivatives

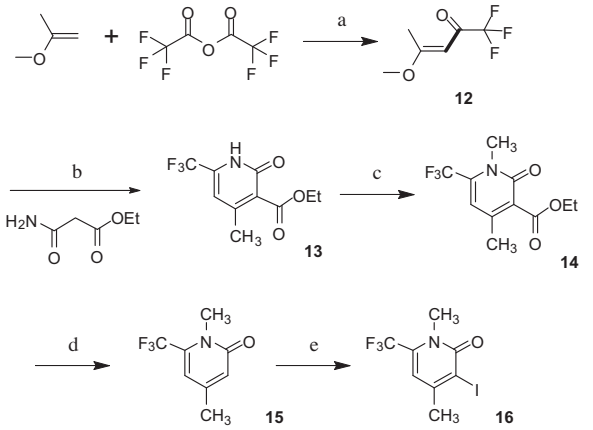


No.	R_1	R_2	R_3	R_4	α4β1 Ramos cells/VCAM IC_{50} nM (Rel Potency)[a]	α4β7 RPMI cells/MadCAM IC_{50} nM (Rel Potency)[a]
23		CH_3	H	H	419	ND
24		CH_3	H	H	582 (0.03)	687 (0.46)
25		CH_3	H	CH_3	226 (0.23)	363 (0.68)
26		CH_3	H	CH_3	90 (0.37)	179 (1.6)
27		CH_3	CH_3	CF_3	48 (0.47)	154 (3.5)
28		CH_3	CH_3	CF_3	32 (0.81)	42 (6.5)
29		H	CH_3	CF_3	820 (0.08)	810 (0.65)
30		C_2H_5	CH_3	CF_3	112 (0.40)	61 (1.9)
31			CH_3	CF_3	463 (0.03)	1230 (0.34)
32		CH_2–C_5H_6	CH_3	CF_3	560 (0.12)	600 (0.96)
33		CH_3	CH_3	CF_3	225 (0.41)	767 (0.70)
34		CH_3	CH_3	CF_3	366 (0.36)	1,290 (0.23)
35		CH_3	CH_3	CF_3	140 (0.75)	671 (0.54)
36		CH_3	CF_3	CH_3	269 (0.11)	126 (2.2)
37		CH_3	CF_3	CH_3	267 (0.06)	160 (2.6)

[a] Rel potency refers to activity relative to the reference standard RO0270608 run as a positive control in each binding experiment.



Scheme 1. Reagents and conditions: (a) TMSCl, H_2O, 0 °C, 15 h; (b) piperidine, EtOH, reflux, 15 h; (c) LiOH, THF, MeOH, reflux, 48 h; (d) $NaHCO_3$, I_2, KI, MeOH, H_2O, 65–70 °C, 15 h; (e) MeI, K_2CO_3, DME, rt, 15 h.



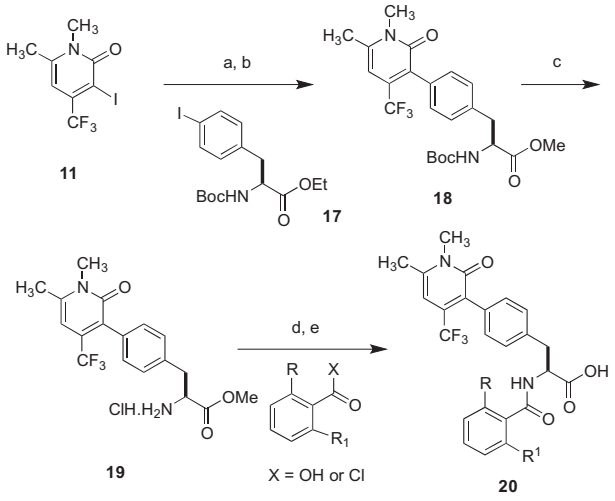
Scheme 2. Reagents and conditions: (a) pyridine, CH_2Cl_2, 0 °C to rt, 15 h; (b) NaOEt, EtOH, reflux, 15 h; (c) MeI, K_2CO_3, DME, reflux, 15 h; (d) LiCl, DMF, 160C, 19 h; (e) NIS, trifluoroacetic anhydride, TFA, 70–85 °C, 2 h.

The 4,6-disubstituted 3-iodo-pyridone intermediates (**11** and **16**) were prepared using the process described in Schemes 1 and 2 while others were prepared using similar approach. In both, a 4,6-disubstituted pyridone core was built by a condensation between the mono ester malonamide and the diketone **7** or the related methoxy vinylketone **12** to give **8** and **13**, respectively. Treatment of the acid **9** with iodine/KI/$NaHCO_3$ in aqueous methanol at 65 °C led to a nearly quantitative conversion to the iodide **10**, which was then alkylated to give **11**.

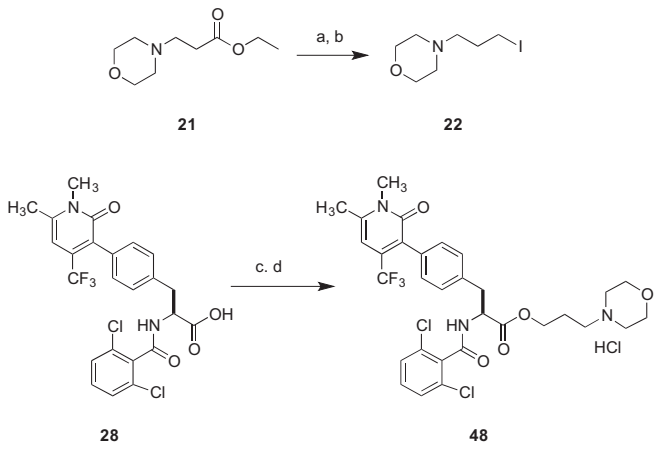
Surprisingly, the carboxylic acid derived from **13** did not provide the corresponding iodide under similar conditions. Thus, **13** was converted to the *N*-methyl intermediate **14** which was then treated with lithium chloride in DMF at 160 °C to effect hydrolysis and decarboxylation followed by treatment with NIS in trifluoroacetic acid in the presence of trifluoroacetic anhydride to give **16** exclusively in 62% yield.

The iodide **11** was then reacted with an activated zinc dust in dimethylacetamide at 70 °C to give the corresponding organozinc intermediate.[8] The coupling reaction of this organozinc intermediate with **17** proceeded smoothly in the presence of $Pd(dba)_2$ and trifurylphosphine (TFP) in THF at 50 °C to obtain the desired product **18** in 77% isolated yield. The remaining steps, BOC removal, benzoylation, and hydrolysis proceeded in a straightforward manner to obtain the target compounds.

In general, the alkyl ester prodrugs were prepared by treatment of the carboxylic acids with the appropriate alkyl iodide or alkyl bromide in the presence of sodium bicarbonate in DMF. For example, the preparation of morpholinopropyl iodide and the corresponding ester prodrug and its HCl salt is shown in Scheme 4.



Scheme 3. Reagents and conditions: (a) Zn dust (activated using 10 mol % dibromoethane and 10 mol % TMSCl in THF), DMAC, 70 °C, 15 h; (b) $Pd(dba)_2$, TFP, THF, 50C, 15 h; (c) 4.0 N HCl in dioxane, dioxane, rt, 5 h; (d) X = OH, HBTU, DIPEA, DMF, rt, 15 h or X = Cl, DIPEA, CH_2Cl_2, rt, 15 h; (e) 1.0 N NaOH, EtOH, rt, 5 h.



Scheme 4. Reagents and conditions: (a) LAH, THF, rt, 2 h; (b) NaI, $MeSO_3H$, CH_2Cl_2, reflux, 15 h; (c) $NaHCO_3$, DMF, rt, 48 h; (d) TMSCl, ^{i}PrOH, rt, 2 h.

The in vitro potency of the compounds listed in Table 1 was assessed by determining the ability of serial dilutions to inhibit the binding of RAMOS cells (α4β1-specific binding) and RPMI 8866 cells (α4β7-specific binding) with recombinant human VCAM-1 used as the counter ligand for the RAMOS cell assay and human MAdCAM-1 for the RPMI 8866 cell assay. Cells were labeled with Calcein AM, a fluorescent dye, and then activated with a binding buffer containing Mn^{2+} to achieve maximum activation prior to assay. RO0270608 (**1**) was used as a positive control on each plate. The data in Table 1 are reported both as the observed IC_{50} and as fold-potency relative to **1** since the activity of the cell lines varied over time with the number of passages.

Comparing the relative potencies of the compounds reported in Table 1 in the cell based assay with the reference compound **1** indicates that **28** is nearly equally potent to **1** against α4β1 and is several-fold more potent as an antagonist of α4β7. The data indicate that R_2 is preferably $-CH_3$ and that R_4 is preferentially trifluoromethyl group relative to $-CH_3$ or H. These findings are consistent with our previously reported observation with 4-benzoylcarbonylaminophenylalanines related to **1** that an electron deficient distal aromatic ring was favorable for potency[9] and esptablished that R_1 is preferably a 2,6-dichlorobenzoyl group.[10] Since **28** met our criteria for potency and selectivity as an α4β7 antagonist, it was

selected for further profiling. Intravenous PK experiments in rat (Table 3) indicated that **28** had an acceptable PK properties.

Previous experience with related compounds indicated that the free acids in the acylphenylalanine class have very poor bioavailability; thus, a prodrug strategy was pursued from the outset. The ethyl ester **38** is extremely insoluble in water (6 μg/mL) and while bioavailability in rats was a modest 18%, it was only 2% in dogs. Other candidate prodrugs were assessed for Caco-2 permeability, solubility, and stability in simulated intestinal fluid, and promising compounds were further profiled in rat PK (Tables 2 and 3). Most of the prodrugs have good stability and reasonable conversion in plasma to the parent drug. Only the basic prodrugs had good solubility and of these, the morpholinopropyl ester **48** emerged as the best candidate for further profiling in large animal PK and efficacy experiments.

PK data for **48** in rat, dog and monkey are shown in Table 3. Although oral bioavailability was only modest in rat and dog, the relatively high bioavailability in monkey, combined with its good solubility and permeability, prompted us to test this compound further in the pivotal monkey lung inflammation model. This model was used to profile **1**, which was validated clinically, and thus became our major in vivo hurdle for taking compounds forward.

Thus cynomolgus monkeys were challenged with aerosolized Ascaris suum extract 2 h after dosing. Inflammatory cell accumulation in the lungs was assessed by examination of bronchiolar lavage fluid withdrawn 24 h after the antigen challenge. At oral doses of 10 and 30 mg/kg po, **48** was efficacious in blocking influx of eosinophils, lymphocytes, neutrophils and total leukocytes into their lungs as shown in Figure 1. Based on its in vivo profile, **48** was selected for preclinical safety studies.

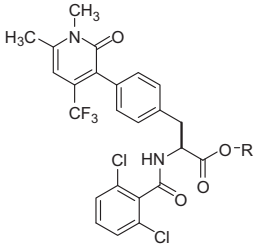
Table 3
PK properties of compounds **28** and **48**

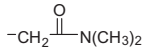
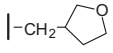
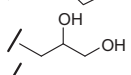
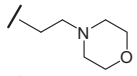
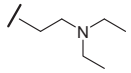
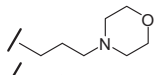
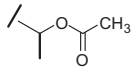
Compd		Rat	Dog	Monkey
28	Dose (iv) (mg/kg)	10	10	15
	AUC (ng h/mL)	10,860	10,880	35,100
	Cl (mL/min/kg)	15.7	16.5	3.0
	V_{ss} (mL/kg)	320	250	67
48	Dose (po) (mg/kg)	50	50	30
	AUC of **28** (ng h/mL)	6100	6420	30,150
	C_{max} (mg/mL)	2.5	2.4	5.0
	F (%)	11	13	46

Surprisingly, although **48** was readily hydrolyzed in mouse, rat and monkey liver microsomes, ester hydrolysis was slow in dog and human. A graphic representation of the microsomal results are shown in Figure 2. The significance of these findings was highlighted by our observation that there was about 10% unchanged prodrug in dog plasma on days 1 and 6 during subchronic safety studies. This gave rise to concerns about tissue distribution and possible safety issues associated with significant exposure to the unchanged prodrug, particularly as it could be anticipated to occur in humans as well.

These concerns were validated when, metabolism studies of radiolabeled **28** and **48** in liver microsomes from rat, dog, monkey and human revealed that significant covalent protein binding occurred with an unknown metabolite derived from the unchanged

Table 2
Prodrug esters of the lead compound **28**



Compd	R	Caco-2 Papp 10^{-7} cm/s	Sol. SGF[a] μg/mL	Sol. SIF[b] μg/mL	Stability SIF[b] $t_{1/2}$ min	hERG IC_{20} μM	Bioavail rat F (%)	Comment
28	–H (Parent)	1.4	—	—	—	—	—	—
38	$-CH_2CH_3$	271	BQL	BQL	Stable	2.7	18	Dog F = 2%
39	$-CH_2CH_2CH_3$	526	BQL	BQL	Stable	nd	nd	
40	$-CH_2CH(CH_3)_2$	325	BQL	BQL	Stable	nd	nd	
41	$-CH_2CH_2OCH_3$	499	BQL	BQL	Stable	nd	nd	
42	$-(CH_2)_3OCH_3$	228	nd	nd	nd	nd	2	
43		63	0.8	0.7	Stable	nd	nd	
44		764	0.03	0.03	Stable	nd	nd	
45		18	190	140	Stable	nd	6	Unchg prodrug in plasma
46		162	>13,000	200	240	11	15	Unchg prodrug in plasma
47		96	>5000	Decomp	30	11.7	12	
48		165	3900	330	Stable	2.9	11	
49		275		4	Stable		17	Mixture of diastereomers

[a] SGF is simulated gastric fluid, pH 2.0.
[b] SIF is simulated intestinal fluid, pH 7.5.

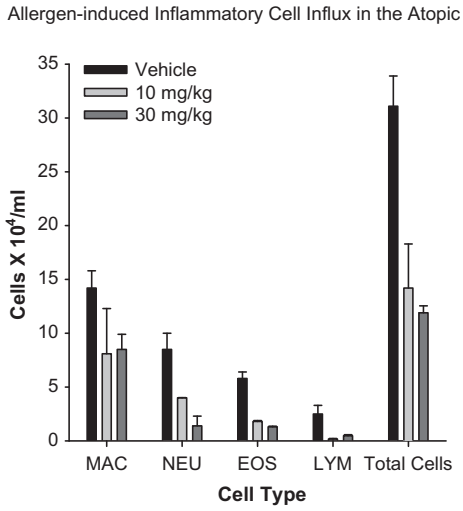


Figure 1. Accumulation of inflammatory cells in atopic non-human primate BAL fluid 24 h after challenge with Acaris suum 2 h after oral treatment with **48**.

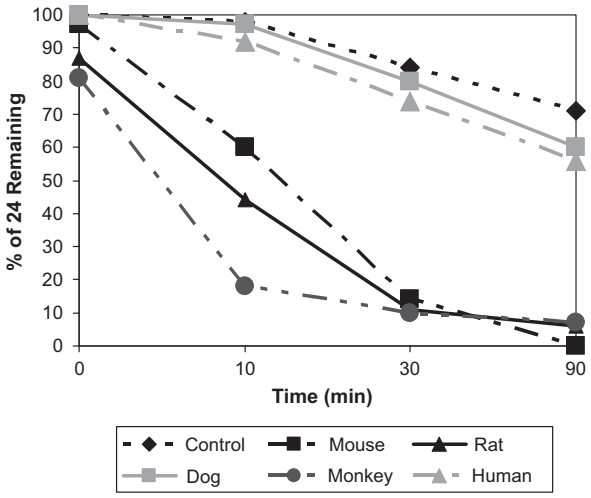


Figure 2. Percent of ester hydrolysis of **48** versus time in liver microsomal preparations from various species. Control refers to incubation of **48** in buffer with no microsomes added.

prodrug, but not the active pyridone acid **28** in dog and human, but not rat or monkey. It is likely that exposure to **48** in the latter was too transient to observe this metabolite or that the metabolic pathway simply was not operable in these species.

In the work presented in this manuscript, a series of pyridones was prepared seeking alpha4 integrin antagonists with a bias for the inhibition of $\alpha4\beta7$. Compound **28** stood out among those investigated and a series of prodrugs was investigated to find a suitable derivative for oral delivery of the otherwise poorly absorbed carboxylic acid. Most compounds investigated were rejected due to poor solubility, permeability or oral bioavailability and attention was focused on the morpholinopropyl ester **48** as having the best combination of in vitro properties and reasonable bioavailability, particularly in monkeys. The unanticipated species selective hydrolysis of **48** led to the abandonment of this compound as a lead candidate and raises a significant cautionary flag for others considering a prodrug approach to solving drug delivery issues.

Scientists interested in pursuing prodrug strategies should verify that any prodrugs they contemplate undergo sufficiently rapid conversion in all species used in pharmacological and safety studies. A similar incidence of species-selective prodrug ester hydrolysis was observed in a series of structurally similar amino acid derived beta2 integrin antagonists.[11] Even though many esters undergo rapid hydrolysis and are rarely employed as drugs per se for this reason, their consistent hydrolysis cannot be taken as a given. It would be interesting to investigate more systematically, how common similar species effects occur. One could, for example examine the time course of hydrolysis of a series of known prodrug esters in liver microsomes and plasma from several species seeking structural patterns. In a forthcoming paper, we will describe a successful prodrug strategy with a series of closely realted pyrimidine-diones in which this phenomena did not occur.[12]

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