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NBD-labeled derivatives of the immunomodulatory drug FTY720 as tools for metabolism and mode of action studies

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Abstract—Fluorescently labeled chiral analogs of the immunomodulatory drug FTY720 and its corresponding phosphates with variable aliphatic spacers between the aromatic ring and the NBD label have been synthesized. Determining the influence of the spacer on the in vitro phosphorylation rate by SPHK1 and 2 resulted in the identification of NBD-(*R*)-AAL **1c**,**d** which are phosphorylated with an efficiency comparable to that of the unlabeled FTY720 analog (*R*)-AAL. Furthermore, the NBD-(*R*)-AAL phosphates **10c**,**d** were proven to be a functional S1P receptor agonist.

FTY720 is a potent immunomodulatory agent with potential usefulness in the control of organ transplant rejection and for treatment of autoimmune diseases such as autoimmune diabetes, multiple sclerosis, and systemic lupus erythematosus.[1,2] FTY720 functions as a prodrug that needs to be phosphorylated by sphingosine kinases in vivo with the resulting FTY720 phosphate being the active principle.[3,4] FTY720 phosphate functions as an agonist at four of the five sphingosine-1-phosphate (S1P) receptors (S1P1, S1P3, S1P4, and S1P5).[5,6] Recent reports propose that binding of FTY720 phosphate to the S1P1 receptor on T-cells leads to receptor internalization and concomitant down regulation, thereby blocking direct migration of T-cells toward high levels of S1P in the bloodstream resulting in inhibiting the egress of T-cells from lymphoid organs.[7] In addition, alternative mechanisms are under investigation.[8]

For further understanding of the mode of action, subcellular localization, and metabolism of both FTY720 and its phosphate, fluorescently labeled, bioactive analogs would be ideal tools. Recently, we and others reported on the synthesis and successful application of pyrene ester- and NBD-labeled sphingosine derivatives.[9,10]

We now report on the synthesis and characterization of nitrobenzo-2-oxa-1,3-diazole- (NBD) labeled chiral FTY720 derivatives based on the functional FTY720 analog (*R*)-AAL[5] with variable lengths of the aliphatic chain between the NBD label and the aromatic ring. The (*R*) enantiomer was chosen because it had been shown previously that (*S*)-AAL is not a substrate of SPHK and that the corresponding (*S*)-phosphate does not signal through S1P receptors[5] (Fig. 1).

Using recombinant human SPHK1 and 2, we describe the substrate properties of these labeled (*R*)-AAL derivatives by quantifying the conversion rate to the corresponding phosphates. In addition, the S1P receptor

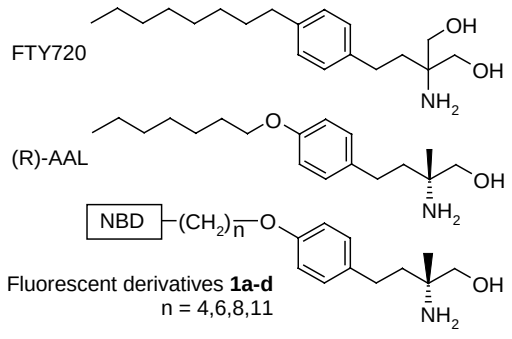

Figure 1. Design of fluorescently labeled (*R*)-AAL derivatives.

Keywords: Immunomodulation; Fluorescence; Sphingolipids; Kinase; Receptors.
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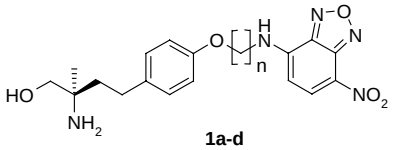
binding profile and functional activity of the corresponding phosphates were determined.

The key intermediate **2** was prepared by the Schoellkopf protocol as recently described.[11] Alkylation of **2** with the commercially available. ω-Bromo-alkylphthalimides **3a**,**b** provided the orthogonally protected amino alcohols **5a**,**b** in 28–32% yield (Scheme 1). The relatively low yield is due to partial hydrolysis of the phthalimide group. Utilizing the dibenzyl-protected mesylates **4c**,**d** under identical alkylation conditions provided **6c**,**d** in 71–76% yield. Deprotection of **5a**,**b** using hydrazine in ethanol provided the primary amines **7a**,**b** in quantitative yield. Selective removal of the dibenzyl-protecting groups by refluxing **6c**,**d** and 10% Pd/C in a methanolic ammonium formate solution provided **7c**,**d** in quantitative yield. **7a**–**d** were reacted with NBD–Cl in THF/TEA to provide **8a**–**d** (70–75% yield). Acidic cleavage of the Boc group provided the NBD-labeled (*R*)-AAL derivatives **1a**–**d**[12] in quantitative yield.

The NBD-labeled (*R*)-AAL derivatives **1a**–**d** were used as substrates for human recombinant SPHK1 and 2.[9] The rates of phosphorylation were determined by assessing the incorporation of radiolabeled phosphate upon incubation with [γ-^{32}P]ATP and the enzymes. Results are reported in Table 1 relative to the natural substrate sphingosine. All FTY720 derivatives were more efficiently converted by SPHK2 compared to SPHK1. As observed already with the fluorescently labeled sphingosine derivatives, conversion rates improved with the length of the aliphatic chain between the head group and the NBD label. While **1a** is not a substrate for SPHK1 and SPHK2, phosphorylation rates for **1b**,**c** are almost comparable to those of FTY720 and (*R*)-AAL. The phosphorylation of **1d** by SPHK2 in vitro was even superior to the natural substrate sphingosine.

Table 1. Rates of phosphorylation of NBD-labeled (*R*)-AAL derivatives by SPHK1 and 2



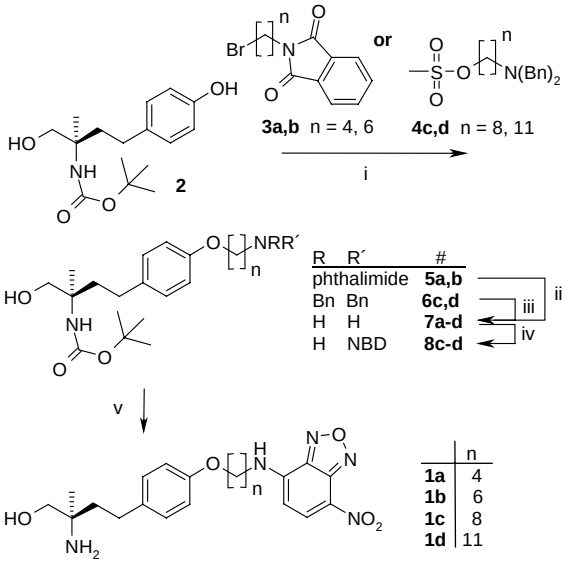
1a-d

Compound	*n*	SPHK1 (%)	SPHK2 (%)
Sphingosine	—	100.0	100.0
FTY720	—	0.6	14.1
(*R*)-AAL	—	4.3	22.9
1a	4	<0.1	<0.1
1b	6	0.7	9.0
1c	8	1.9	6.5
1d	11	5.6	119.0

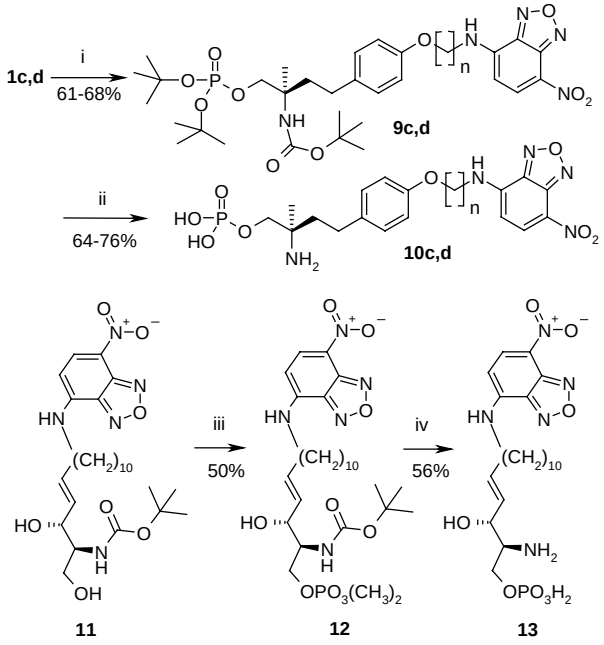
Values are given relative to the rate for D-sphingosine.[9]

We selected amino alcohols **1c**,**d** for further chemical conversion to the corresponding NBD-labeled phosphates (*R*)-AAL-P (**10c**,**d**). Thus, **10c**,**d**[13] were obtained in good yield using standard phosphoamidate chemistry (Scheme 2).

To check whether the NBD label would affect the S1P receptor binding profile, we also prepared the NBD-labeled S1P derivative **13** using the trimethyl phosphite procedure described by Hakogi et al.[10] (Scheme 2). After experiencing some difficulties in isolating **12** in reasonable yield, we found that the phosphorylation of the Boc-protected NBD-sphingosine **11**[9] worked best by adding 2.2 equiv of CBr_4 and 2.6 equiv of trimethylphosphite to a solution of **11** in a minimum amount



Scheme 1. Variation of the label. Reagents and conditions: (i) K_2CO_3, EtOH/DMF 3/1, 50 °C; (ii) $N_2H_4{\cdot}H_2O$, EtOH, reflux; (iii) 10% Pd/C, ammonium formiate, MeOH, reflux; (iv) NBD–Cl, Et_3N, THF; (v) 10% aq TFA, rt.



Scheme 2. Preparation of the labeled phosphates. Reagents and conditions: (i) (a) 1*H*-tetrazole, di-*tert*-butyl-diethylphosphoramidite, THF, rt, (b) H_2O_2; (ii) 10% aq TFA, rt; (iii) CBr_4, pyridine, $P(OCH_3)_3$, −10 °C to rt; (iv) TMS-Br, acetonitrile, rt.

of dry pyridine at −10 °C and subsequently allowing the reaction mixture to warm to room temperature.

Motivated by the good in vitro phosphorylation of **1c**,**d** by SPHK2, we investigated the S1P-receptor binding profile of the corresponding NBD-labeled (*R*)-AAL phosphates **10c** and **d** in comparison to that of (*R*)-AAL phosphate, S1P and NBD-labeled S1P **13** (Table 2). The [γ-^{35}S]GTPγS binding assays were performed as described previously.[5] NBD-S1P (**13**) was found to be an agonist for all S1P receptors tested. While its potency (pEC_{50}) is 1–2 log steps lower compared to that of unlabeled S1P, it is still in the nanomole-range. The efficacy (E_{max}) is reduced, especially for the S1P1 receptor. The NBD-labeled (*R*)-AAL-phosphates **10c**,**d** also functioned as agonists at the S1P receptors with slightly reduced potency and efficacy compared to that of the unlabeled derivative. As previously shown for (*R*)-AAL-phosphate,[5] **10c**,**d** do not signal via the S1P2 receptor (data not shown).

In line with the binding data, **10c**,**d** induced Ca^{2+} mobilization in S1P1 and S1P3 overexpressing CHO cells.[14] For the S1P1 receptor the potency and efficacy was comparable to that of S1P and was significantly weaker for the S1P3 receptor (Fig. 2). Taking both the S1P1 binding data and the Ca^{2+} mobilization assay into account, **10c** and **d** were found to be equally potent and effective functional S1P receptor agonists.

We used the labeled amino alcohol **1d** to examine the uptake, subcellular distribution, and metabolism (conversion to the phosphate) in human endothelial cells (HUVEC). Fluorescently labeled (*R*)-AAL **1d** was rapidly incorporated into the cells within 5 min after addition and showed predominant distribution to the endoplasmic reticulum. The plasma membrane and, in some cells, mitochondria were visibly labeled as well (Fig. 3A). **1d** was converted intracellularly to the phosphate **10d** as shown by thin-layer chromatography[9] (Fig. 3B). The conversion efficiency was comparable to that of FTY720 (data not shown). These data indicate that the NBD label does not hinder enzymatic phosphorylation within cells.

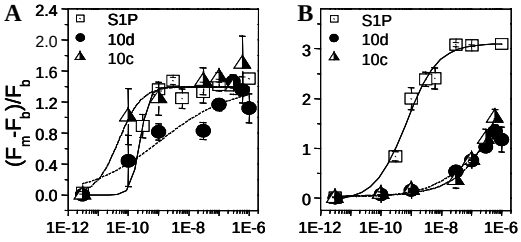


Figure 2. Ca^{2+} mobilization by **10c** and **d** compared to that of the endogenous agonist sphingosine-1-phosphate (S1P). CHO cells expressing S1P1 (A) and S1P3 (B) were treated with different concentrations of the agonists and signals were recorded by a fluorescent image plate reader. Plotted is molar concentration against fold change in fluorescence.[14]

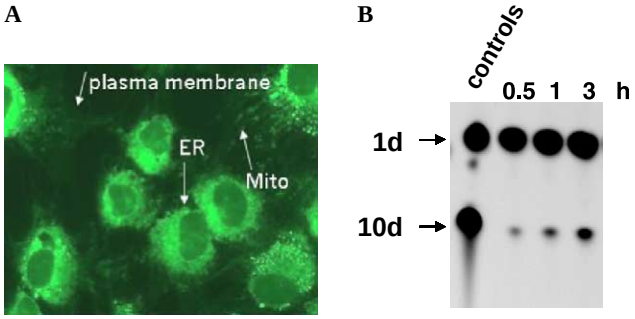


Figure 3. (A) Rapid incorporation of **1d** into endothelial cells (HUVEC). Cells were incubated for 15 min with 1 μM **1d** under normal growth conditions; ER, endoplasmic reticulum; Mito, mitochondria. (B) Metabolic conversion of **1d** assayed by thin-layer chromatography. Cells were incubated for 0.5, 1, and 3 h with 1 μM **1d** followed by lipid extraction.

Because of the superior aqueous solubility of **10c**[15] compared to **10d** in cell culture medium, we used the NBD-labeled (*R*)-AAL phosphate **10c** and NBD-S1P **13** to examine the MAP kinase/ERK activation in HUVEC.[16] At 1 μM, both NBD-labeled agonists were able to trigger ERK phosphorylation (Fig. 4) with an efficiency comparable to that of unlabeled FTY720 phosphate.

Table 2. Potency (pEC_{50}) and efficacy (E_{max}) values of phosphorylated compounds at human S1P receptors

Receptor	Parameter	Compound				
		S1P	NBD-S1P **13**	(*R*)-AAL-P	**10c**	**10d**
S1P1	pEC_{50}[a]	9.0 (8.1–9.7)[b]	7.2 (7.0–7.3)	9.1 (8.9–9.2)	7.7 (7.4–7.9)	9.0 (8.7–9.3)
	E_{max}	1.00	0.67 (0.58–0.76)	0.91 (0.80–1.00)	0.76 (0.74–0.78)	0.82 (0.76–0.87)
S1P3	pEC_{50}	9.75 (9.1–10.5)	8.3 (8.1–8.4)	8.2 (8.0–8.5)	7.7 (7.5–7.9)	7.5 (7.2–7.8)
	E_{max}	1.00	0.99 (0.78–1.20)	0.43 (0.32–0.49)	0.87 (0.68–1.05)	0.69 (0.56–0.82)
S1P4	pEC_{50}	8.9 (8.1–9.5)	7.2 (7.0–7.4)	8.9 (8.1–9.5)	7.7 (7.4–7.9)	[d]
	E_{max}	1.00	1.06 (1.02–1.10)	1.53	0.27 (0.16–0.37)	[d]
S1P5	pEC_{50}	8.7 (8.4–9.1)	7.3 (7.1–7.6)	8.7 (8.4–9.1)	[c]	8.7 (8.3–9.1)
	E_{max}	1.00	0.80 (0.71–0.88)	0.75 (0.53–1.00)	[c]	0.27 (0.23–0.31)

[a] pEC50, −log molar concentration of compounds resulting in 50% of maximal GTPγS binding; E_{max}, maximal [γ-^{35}S] binding as a fraction of the S1P signal (set at 100% = 1.0).
[b] Average value of three to six experiments (range of values).
[c] The shape of binding curve did not allow the calculation of an EC_{50}.
[d] Not an agonist up to 10 μM.

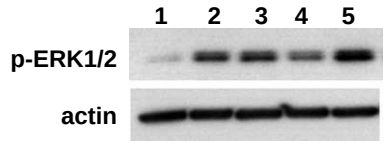


Figure 4. Activation of ERK1/2 by NBD-S1P **13** (2), NBD-(*R*)-AAL phosphate **10c** (3), S1P (4) and FTY720 phosphate (5) in endothelial cells. Short-starved cells were incubated for 10 min with 1 μM of compound and collected for Western blot analysis. Membranes were incubated with antibodies directed to phosphorylated ERK1/2 and re-probed with anti-β-actin antibodies to ensure equal loading of the samples; 1, unstimulated cells; 2–5, compounds as indicated in brackets.

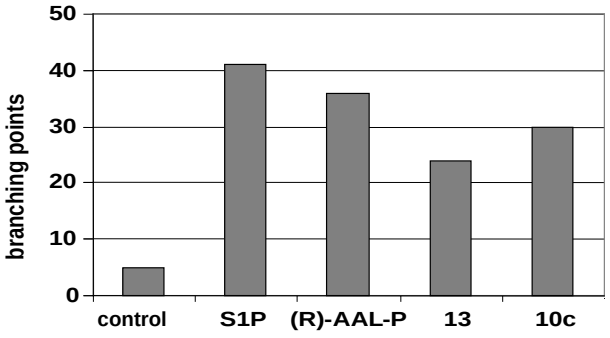


Figure 5. Activity of test compounds at 1 μM in an in vitro angiogenesis model. The capillary-like network formation on Matrigel upon activation of HUVEC for 8 h is shown. Effects were quantitated by a direct counting of the number of branching points per microscopic field.

To further characterize the biological activities of NBD-labeled phosphates in a cellular system, we investigated the effect of NBD-(*R*)-AAL phosphate **10c** and NBD-S1P **13** on morphogenic differentiation of endothelial cells using an in vitro angiogenesis assay on Matrigel,[16,17] wherein the number of branching points reflects the potency of the stimulus. As shown in Figure 5, both unlabeled (*R*)-AAL phosphate and S1P and their NBD-labeled analogs **10c** and **13** strongly promoted the capillary-like network formation.

In summary, we prepared fluorescence-labeled chiral FTY720 analogs and their corresponding phosphates, which can serve as valuable tools for many biological investigations. Amino alcohol derivatives with an octanyl (**1c**) or undecanyl (**1d**) spacer between the NBD-label and the phenoxy ring were proven to be efficiently phosphorylated by SPHK2 in vitro and in vivo. **1d** was rapidly taken up by cells and was distributed preferentially to the endoplasmic reticulum. The corresponding phosphates **10c**,**d** were proven to be potent and efficacious agonists for the S1P receptors **10c**,**d** induced Ca^{2+} mobilization in S1P1 and S1P3 overexpressing CHO cells comparable to that of the endogenous ligand S1P. Furthermore, the NBD label did not interfere with ERK activation and the pro-angiogenesis effect of S1P and FTY720.

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12. Characteristic data for **1d**: ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 9.53 (br s; 1H), 8.48 (d; J = 8.9 Hz; 1H), 7.75 (br s; 3H), 7.05 (d; J = 6.6 Hz; 2H), 6.81 (d; J = 6.6 Hz; 2H), 6.38 (d; J = 8.9 Hz; 1H), 5.50 (br s; 1H), 3.86 (t; J = 6.5 Hz; 2H), 3.43 (m; 2H), 2.50 (m; 2H), 1.80–1.60 (m; 6H), 1.42–1.15 (m; 14H), 1.18 (s; 3H). Electrospray MS: 528.3 $(M+H)^+$, $C_{28}H_{41}N_5O_5$, calcd 527.3.
13. Characteristic data for **10d**: ^{1}H NMR (CD_3OD + 1 drop DCL, 500 MHz): δ = 8.51 (d; 1H), 7.13 (d; 2H), 6.81 (d; 2H), 6.34 (d; 1H), 4.09/4.01 (ABX system, 2H), 3.91 (t; 2H), 3.54 (br s; 2H), 2.63 (m; 2H), 2.04–1.87 (m; 2H), 1.81–1.69 (m; 2H), 1.50–1.29 (m; 14H), 1.41 (s; 3H). Electrospray MS: 608.4 $(M+H)^+$, $C_{28}H_{42}N_5O_8P$, calcd 607.28.
14. CHO overexpressing S1P receptors were plated in black Costar plate. Cells were loaded with 2 μM Fluo4AM (Molecular Probes), 5 mM probenecid for 1 at 37 °C, rinsed, and transferred to the fluorescent image plate reader (FLIPR). The cells were pre-treated for 15–25 min with 10 μM ATP and, after measuring the baseline fluorescence (F_b) for 40 s, the agonist was added to determine the peak of the fluorescence F_m (3–5 min). Pretreatment with ATP allows efficient coupling of S1P receptors to Ca^{2+} mobilization as described for other GPCRs by Werry et al. *Biochem. J.* **2003**, *374*, 281.
15. A 10 mM stock solution of **10c** in DMSO/1 N HCl (15/2 v/v) is diluted with assay medium.
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