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Synthesis and evaluation of some lipidic aminoalcohols and diamines as immunomodulators

Esther del Olmo,[a,*] Alvaro Plaza,[b] Antonio Muro,[b,*] Antonio R. Martínez-Fernández,[c] Juan J. Nogal-Ruiz,[c] José L. López-Pérez[a] and Arturo San Feliciano[a]

[a]*Departamento de Química Farmacéutica, Facultad de Farmacia, CIETUS, Universidad de Salamanca, 37007 Salamanca, Spain*
[b]*Laboratorio de Parasitología, Facultad de Farmacia, CIETUS, Universidad de Salamanca, 37007 Salamanca, Spain*
[c]*Departamento de Parasitología, Facultad de Farmacia, Universidad Complutense, 47007 Madrid, Spain*

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Abstract—Lymphoproliferation inhibition and cytotoxicity of a number of lipidic aminoacids, aminoalcohols and diamines were evaluated as a preliminary screening to select potential immunomodulators. The four most potent/less toxic compounds were submitted to delayed hypersensibility (DTH) assays to define the best to be evaluated further Graft-vs-Host, NO production and other immunoevaluation ($CD4^+$, CD45, CD8, CD11b, I-Ek, and NK cells) assays, to establish their immunomodulation potential for being further considered as auxiliary agents for vaccination against some parasitic infections. Compounds **5d**, **6d**, **6f**, **7a**, and **9a**, fairly inhibited the lymphoproliferation (71.6–79.5%, at 3.2–2.4 nM), while the aminoalcohol derivative **6f** and the diamine **7a** gave the most promising results in the DTH assays. Diamine derivative **8b** induced nitrite production on normal macrophages, whereas compounds **6f** and **7a** induced nitrite production on LPS pre-stimulated macrophages. These two last compounds have been selected to follow in vivo vaccination assays.

Immunosuppressants play important clinical roles in organ transplantation and in the treatment of autoimmune diseases such as rheumatoid arthritis, psoriasis and systemic lupus erythematosus. Cyclosporin A (CsA)[1] and tacrolimus (FK506)[2] have made great contributions to the prevention of acute rejection in human organ transplantation. Both drugs have similar mechanisms of action and proved their immunosuppressant activity by inhibiting the production of interleukin-2 (IL-2) in antigen-stimulated helper T-cells,[3–5] but they also have severe side effects,[6] such as a high cytotoxicity and renal and liver toxicities. Therefore, less toxic drugs for the prevention of graft rejection are needed.

ISP-1 (myriocin, thermozymocidin) was isolated in 1989 from the culture broth of *Isaria sinclairii* by Fujita et al.[7] These authors also described the ISP-1 immunosuppressant activity,[8] that was nearly five times more potent than that of CsA, with lower toxicity. They also worked on the identification of the minimal structure requirements for the activity and concluded that a 2-alkyl-2-aminoethanol moiety was needed.[9] They also optimised the alkyl chain length, the functions and their locations on the alkyl chain to develop compound FTY720 (IC_{50} 6.1 nM), as the best drug candidate.[10] In the course of these studies, compound OA-12 displayed an intermedium immunosuppressant activity (IC_{50} 98.3 nM), nearly seven times lesser potent than CsA in the allogeneic mouse mixed lymphocyte reaction (MLR) assay.[11]

Our research group has been working on lipidic ethylenediamine and β-aminoalcohol derivatives displaying different biochemical, pharmacological and antimicrobial properties.[12–16] The close structural relationship between the above-mentioned drugs and compound **OA-12** with some related compounds prepared by us suggested their evaluation as immunomodulators through lymphoproliferation inhibition, delayed hypersensitivity and other studies.

Keywords: Synthesis; Evaluation; Lipidic aminoalcohols; Lipidic diamines; Immunomodulators.
* Corresponding authors. Fax: +34 923294515; e-mail: olmo@usal.es

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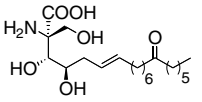
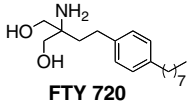
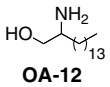


ISP-1
(myriocin, thermozymocidin)



FTY 720



OA-12

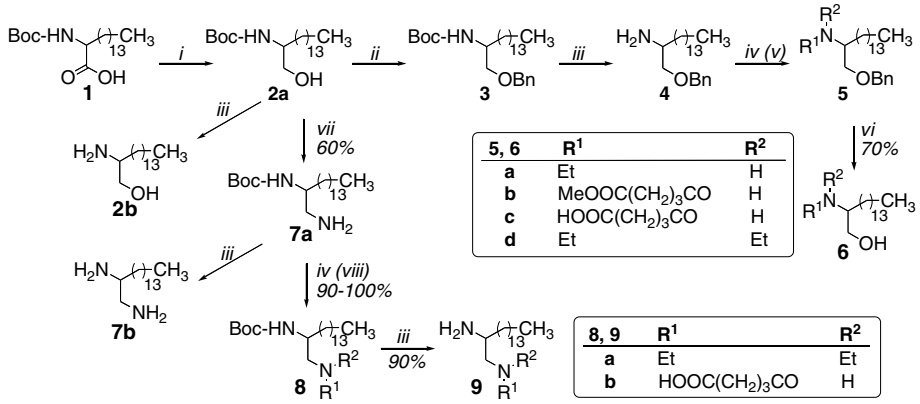
The lipidic compounds being tested in this multi-screening research were obtained as described previously (Scheme 1).[12] The starting aminoacid derivative **1** was prepared[17] from the intermediate diethyl 2-tetradecylacetamidomalonate (obtained by alkylation of diethyl 2-acetamidomalonate in the presence of NaOEt), after refluxing in concd HCl and treatment with Boc anhydride. Compound **1**, transformed into the mixed anhydride by reaction with ethyl chloroformate,[18] followed by reduction with $NaBH_4$, provided the alcohol **2a**. Compound **2a** was transformed into the benzyl ether **3**, then by acid hydrolysis of the Boc-protecting group, into the benzyloxy amine **4**. Amine **4** was transformed into the secondary amine (**5a**) or the tertiary amine (**5d**) by alkylation with ethyl bromide or into the amide derivative **5b**, by acylation with the chloride methylester of glutaric acid. The free acid **5c** was obtained after treatment of **5b** with aq KOH (10%). Hydrogenolysis of the benzyl group of compounds **5a**–**d** with H_2/Pd–C led to the free alcohols **6a**–**d**, respectively.

Diamine derivatives **7**–**9** were obtained from compound **2a**. The primary alcohol was first converted into its mesylate, then into the azide and then reduced with H_2/Pd–C, $NaBH_4$ to give the diamine **7a**, which treated with an excess of ethyl bromide or with one equivalent of glutaric anhydride gave **8a** and **8b**, respectively. Boc removal with HCl(g) in THF gave, respectively, compounds **9a** and **9b**. Similarly the free aminoalcohol **2b** and the diamine **7b** were obtained from compounds **2a** and **7a**, respectively, by treatment with acid.

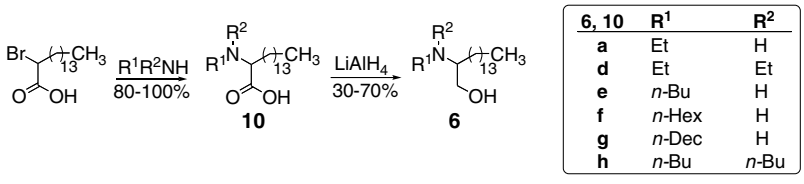
A faster procedure leading to aminoalcohols of type **6** was also applied to obtain another group of aminoalcohol derivatives. Starting from 2-bromohexadecanoic acid and the proper amines, the intermediate aminoacids of type **10** were prepared, from which $LiAlH_4$ reduction yielded the corresponding aminoalcohols **6a** and **6d**–**h** (Scheme 2).

Lymphoproliferation inhibition was assessed on splenocyte cultures stimulated with concanavalin-A (Con-A)[19] and evaluated through the cellular respiration levels determined by a colourimetric MTT assay.[20] Cytotoxicity was determined in parallel. Anapsos®, a hydroalcoholic extract obtained from the rhizome of the American fern *Phlebodium pseudoaureum* (synom.: *Polypodium leucotomos* P., Polypodiaceae), was included in the tests as reference standard. This drug, reported to be clinically useful against neoplasms[21] and autoimmune diseases such as atopic dermatitis,[22] psoriasis[23,24] and vitiligo,[25] might also have some utility in the treatment of allergic disorders[26] and as immunomodulator in vaccine formulation against parasites.[27]

The results of the assays, shown in Table 1, were calculated from colourimetric measures according to the formulas:



Scheme 1. Synthesis of lipidic aminoalcohol and diamine derivatives. Reagents and conditions: (i) a—EtOOCCl/*N*-methylmorpholine/THF; b—$NaBH_4$/MeOH; (ii) BnCl/NaH/DMF; (iii) HCl(g)/THF; (iv) EtBr/Et_3N/DMF; (v) glutaric acid monomethyl ester chloride/ether; (vi) H_2/Pd–C/AcOH; (vii) a—MsCl/Et_3N/CH_2Cl_2; b—NaN_3/DMF; Pd–C/$HCCl_3$; (viii) glutaric anhydride/CH_2Cl_2.



Scheme 2. Synthesis of aminoalcohols of type **6** from 2-bromopalmitic acid.

Table 1. Lymphoproliferation inhibition, cytotoxicity and nitrite production by lipidic aminoacid, aminoalcohol and diamine derivatives

Compound	Lymphoproliferation inhibition				Nitrite production increase	
	10 μg/mL		1 μg/mL			
	Inhibition (%)	Cytotoxcity (%)	Inhibition (%)	Cytotoxcity (%)	Normal machrophages	LPS pre-stimulated machrophages
1	32.9	5.1	nd	nd	nd	nd
2b	72.4	20.2	68.7	15.1	nd	nd
4	79.1	12.2	0	0	ni	ni
5a	49.8	11.9	nd	nd	nd	nd
5b	3.4	2.5	nd	nd	ni	ni
5c	65.4	6.4	27.8	0	ni	ni
5d	72.6	15.6	71.6	14.1	nd	nd
6a	72.9	17.1	68.4	10.5	nd	nd
6b	50.2	18.9	nd	nd	nd	nd
6c	49.1	14.5	nd	nd	nd	nd
6d	79.5	12.8	74.8	7.5	nd	nd
6e	49.1	16.4	nd	nd	nd	nd
6f	78.4	14.6	74.1	5.0	ni	25% (at 100 μg/mL)*
6g	59.8	12.6	12.0	6.6	nd	nd
6h	50.3	11.7	nd	nd	nd	nd
7a	81.2	4.3	73.4	4.2	ni	20% (at 10 μg/mL) 29% (at 100 μg/mL)*
7b	50.1	2.5	nd	nd	nd	nd
8a	89.5	7.8	65.2	6.5	nd	nd
8b	80.8	1.2	19.3	0	+*	ni
9a	84.5	24.2	74.2	1.7	ni	ni
9b	51.0	12.8	nd	nd	ni	ni
10d	43.1	12.1	nd	nd	nd	nd
10e	32.9	5.1	nd	nd	nd	nd
10h	47.8	12.5	nd	nd	nd	nd
Anapsos®	nd	nd	66.3	23.2	ni	ni

nd, no determined; ni, no increase of NO production.
* Statistically significant increase, $p < 0.05$ (ANOVA Fisher test).

$$\text{Proliferation inhibition } (\%) = [1 - (\text{OD}_{\text{cells+ConA+compd}})/(\text{OD}_{\text{cells+ConA}})] \times 100;$$

$$\text{Cytotoxicity}(\%) = [1 - (\text{OD}_{\text{cells+compd}})/(\text{OD}_{\text{cells}})] \times 100;$$

$$(\text{OD} = \text{Optical density})$$

Twenty-four compounds, four aminoacids, nine aminoalcohols, five benzyloxyamines and six diamines, were tested in the lymphoproliferation inhibition assay at a 10 μg/mL concentration initially. Those displaying inhibition values higher than 50% were then tested at 1 μg/mL (Table 1).

Thus, the starting Boc-aminoacid **1**, and the *N*-alkyl- or *N*,*N*-dialkyl-aminoacids **10d**, **10e** or **10h** did not show strong inhibition at 10 μg/mL and were not further tested. Within the group of 2-aminohexadecanol derivatives those having the free primary hydroxyl function, compounds **2b** and **6a**–**h**, displayed fair proliferation inhibitory effects, attaining up to almost 75% inhibition, in the case of compound **6d**. Benzyl ethers **4** and **5** showed greatly reduced potencies in general, as it can be seen for the pairs of compounds **2b/4**; **6a/5a**; **6b/5b**; **6d/5d**, with a not totally clear exception, for the pair **6c/5c**. The number of alkyl groups attached to the amino group influences the potency, but the effect is also dependent on the size of the alkyl chains. Thus, dialkylation of aminoalcohols seems to increase the potency with the number of ethyl groups: **2b** (NH_2) ~ **6a** (NHEt) < **6d** (NEt_2); whereas in the case of butyl derivatives the alkylation reduces considerably the potency: **2b** (NH_2) ≫ **6e** (NHBu) ~ **6h** (NBu_2). For monoalkylamino derivatives there is an optimum C_6-size of the chain: [**2b** < **6a**(Et) > **6e**(Bu) ≪ **6f** (hex) ≫ **6g** (dec)]. Thus, within this series of aminoalcohol derivatives, compound **6d** became the most potent at those concentrations tested. In other sense, the presence of a tertiary amine seems to be better for the activity than that of a secondary one **6d/6a** (79.5/72.9), **6h/6e** (50.3/49.1) and **10h/10e** (47.8/32.9).

Within the small number of 1,2-diamine derivatives tested, **7**–**9**, four compounds displayed lymphoproliferation inhibition values over 80% at 10 μg/mL and two of them over 70% at 1 μg/mL, compound **8a** (79.5% at 10 μg/mL) being the most potent inhibitor. The Boc group on the 2-amino function increases the inhibitory activity, as it can be observed through comparison of the pairs **7a/7b** (81.2/50.1%), **8a/9a** (89.5/84,5%) and **8b/9b** (80.8/51.0%) though the last pair contains another structure change that could invalidate this comparison.

Taking into consideration the results of proliferation inhibition and cytotoxicity, compounds **4**, **6f**, **7a**, and

9a were selected for evaluating their response in the assay of delayed type of hypersensivity (DTH) induced by the crude antigen of *Trichinella spiralis* (TSA).[28] The modulation of the inflammatory response was tested after 24 and 48 h of administration and revealed that compounds **6f** (at 24 h) and **7a** (at 48) increased, but not significantly, the TSA response, while compounds **4** and **9a** decreased it, also with no statistical significance.

In another experiment, some selected four aminoalcohol and four diamine derivatives were evaluated for their ability for influencing nitric oxide production on normal or pre-stimulated rat alveolar macrophages in vitro.[29] As it can be seen (Table 1), the diamine **8b** induced nitrite production in normal macrophage cultures in a dose-dependent manner (data not shown), whereas compounds **6f**[30] and **7a**[31] increased NO production significantly in the LPS pre-stimulated macrophages culture. In those experiments we did not find any increase of nitrite production in normal or pre-stimulated macrophage cultures treated with Anapsos®.

As an additional proof for establishing their immunomodulatory potential, the two selected compounds, **6f** and **7a**, were evaluated through a Graft-vs-Host assay[32] to define their immunosuppresant ability in comparison with cyclophosphamide (Cy, 100 mg/kg/day), taken as the reference drug. The aminoalcohol derivative **6f** significantly reduced the lymphocyte stimulation by some 63%, close to the Cy immunosuppression value of 71%. In the same experimental conditions the diamine **7a** attained also to reduce lymphocyte stimulation by around 32%, though with no statistical significance.

Other in vitro experiments carried out with the selected compounds were focused on immunophenotype characterisation, through analysing changes in the production of a number of immunomodulation relevant cells by flux cytometry. Both compounds **6f** and **7a** increased the percentage of T helper lymphocytes $CD4^+$ or CD8 and I-Ek cells of exposure to antigen, though with no statistical significance (data not shown).

In summary, eight out of those twenty-four compounds evaluated were better than the reference drug Anapsos®, and some of them also more potent than those literature compounds **OA-12** and **FTY720**, for inhibiting lymphoproliferation, with low toxicity. After several immunoevaluation assays, compounds **6f** and **7a** have proven their good qualities, and have been selected to follow further in vivo evaluations as potential adjuvants for vaccination. In order to progress in this research, the racemic resolution of these compounds **6f** and **7a** is being carried out. Additionally, taking into account the reduced number of compounds included in the initial prospective screening, the synthesis and evaluation of larger aminoalcohol an diamine libraries of compounds are currently being considered.

Acknowledgments

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30. Analytical data for compound **6f**. IR ν_{max}: 3600, 3050, 2927, 1522, 1220, 1046 and 928 cm^{-1}. ^{1}H RMN (200 MHz, $CDCl_3$): δ (ppm) 3.62 (1H, dd, $J_1 = 10.2$, $J_2 = 6.9$ Hz, H-1_A); 3.25 (1H, dd, $J_1 = 10.2$, $J_2 = 6.5$ Hz, H-1_B); 2.60 (3H, m, H-2 +N-CH_2); 1.25 [34H, m, $(CH_2)_{13}$ + $(CH_2)_4$]; 0.91, (3H, t, CH_3), 0.87, (3H, t, CH_3). ^{13}C RMN (50.3 MHz, $CDCl_3$): δ (ppm) 62.8, 59.2, 46.9, 31.9, 31.7, 31.3, 30.1, 29.7, 29.4, 27.0, 26.2, 22.7, 14.1 ppm. MS (CI) *m*/*z* = 341,37. Anal. Calcd for $C_{22}H_{47}NO$: C, 77.35; H, 13.87; N, 4.10. Found: C, 77.05; H, 13.52; N, 4.07.
31. Analytical data for compound **7a**. mp: 56 °C. IR ν_{max}: 3349, 2918, 2850, 1687, 1531, 1365, 1349 and 1174 cm^{-1}. ^{1}H RMN (200 MHz, $CDCl_3$): δ (ppm) 4.48 (1H, sa, N*H*-Boc), 3.51 (1H, m, H-2), 2.77 (1H, dd, $J_1 = 12.8$, $J_2 = 7.0$ Hz, H-1_A); 2.61 (1H, dd, $J_1 = 12.8$, $J_2 = 4.0$ Hz, H-1_B); 1.25 [26H, m, $(CH_2)_{13}$]; 1.49, [9H, s, $(CH_3)_3$], 0.88, (3H, t, CH_3). ^{13}C RMN (50.3 MHz, $CDCl_3$): δ (ppm) 155.3, 54.7, 50.4, 33.0, 32.0, 29.7, 29.4, 28.5, 26.1, 22.8, 14.2 ppm. MS (FAB) *m*/*z* = 357,37. Anal. Calcd for $C_{21}H_{44}N_2O_2$: C, 70.73; H, 12.44; N, 7.86. Found: C, 70.65; H, 12.38; N, 7.81.
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