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Synthesis of Non-immunosuppressive Cyclophilin-Binding Cyclosporin A Derivatives as Potential Anti-HIV-1 Drugs

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Dedicated to the memory of Dr. Jean-Claude Barrière

Abstract—Original cyclosporin A (CsA) derivatives bearing various alkylthio side chains at the sarcosine residue 3 (*R* configuration) and for the most potent and selective compounds a 4′-hydroxyl group at the Me-Leucine residue 4 were prepared in one or two steps from commercially available CsA. The [2-(dimethyl or diethylamino)-ethylthio-Sar]^3-[(4′-OH)MeLeu]^4-CsA derivatives **3k** and **3l** displayed potent in vitro anti-HIV-1 (IC_{50} ~46 nM) and low immunosuppressive activities ($IC_{50} \geq 1500$ nM).

The pandemic spread of human immunodeficiency virus (HIV) has promoted an unequalled scientific effort to understand and control this disease. Studies on the life cycle of HIV-1 have resulted in the development of many anti-HIV drugs. Reverse transcriptase inhibitors, typified by AZT, provided the first approved anti-HIV drug. Other nucleosidic reverse transcriptase inhibitors such as Didanosine (ddI), Zalcitabine (ddC), Stavudine (D4T) as well as non-nucleosidic inhibitors appeared later on.[1] Approval of peptidal inhibitors of the viral protease, including Amprenavir, Indinavir, Lopinavir, Nelfinavir, Ritonavir and Saquinavir, has proven invaluable as an addition of the armamentarium of the physician.[2] Administration of cocktail including nucleoside and/or non-nucleoside reverse transcriptase inhibitors, combined with protease inhibitors, allows both the prevention of viral replication and the development of resistance at low, less toxic doses but does not eradicate the latent virus.[3] Despite the success of highly active antiretroviral therapy (HAART), AIDS remains one of the most urgent world health problem and there is still a widely recognized clinical need for additional viral/cellular targets for HIV therapy. Indeed, rapid emergence of drug-resistant HIV variants and severe side effects limit the efficacy of existing therapies. The intrinsic high variability of HIV calls for combining different drugs with distinct mode of action to achieve synergistic antiviral activity. Efforts are being made to develop agents addressing new steps in HIV replication and to optimize both antiviral activity and pharmacokinetic of the current drugs targeting reverse transcriptase and protease.

Cyclosporins, are hydrophobic cyclic undecapeptides produced by the fungus *Tolypocladium niveum*. The main metabolite, Cyclosporin A (CsA, Sandimmune®, Fig. 1) is the first-line drug currently used to prevent the rejection of organ and bone marrow transplants.[4] Seven of its 11 constitutive amino acids are *N*-methylated. Furthermore, three of them do not occur in mammalian proteins: (4*R*)-4-[(*E*)-2-butenyl-4-methyl-L-threonine (MeBmt) in position 1, (L)-α-amino-butyric acid in position 2, and (D)-alanine in position 8.[5]

Cyclosporin A (CsA) and its derivatives [like [Me-Ile]^4-CsA (NIM 811)[6]] that bind cyclophilin A exert anti-

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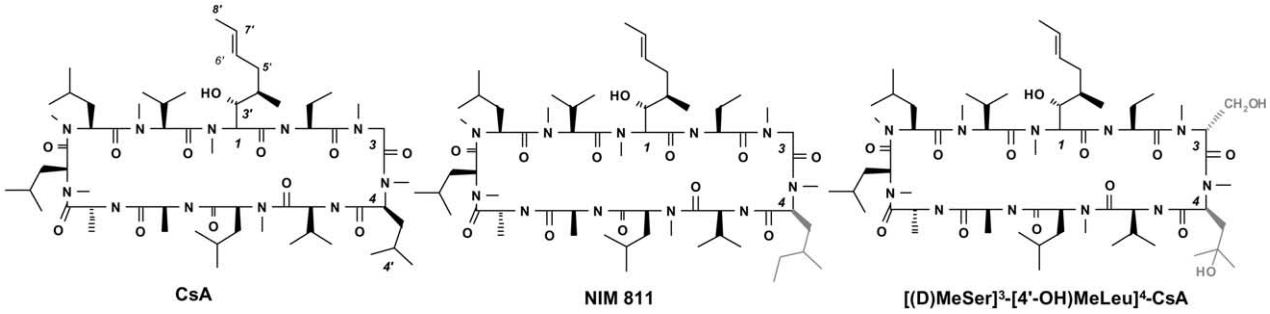


Figure 1.

HIV activity by a mechanism independent of immunosuppression. Cyclophilin A is implicated in viral infectivity via its interaction with p24, a viral core protein. Cyclophilin A is believed to enhance HIV_1 infectivity. CsA and non-immunosuppressive analogues disrupt gag-binding to cyclophilin A, prevent cyclophilin A incorporation into virions, and block the replication of HIV_1 in cells. However, some viral strains, such as those belonging to the HIV_2 subtype, do not require cyclophilin A for replication and therefore would not be affected by CsA.[7] The immunosuppressive properties of CsA is an obvious handicap for its clinical use as anti-HIV inhibitor. Therefore, the search for non-immunosuppressive cyclosporin analogues has been conducted.

Few non-immunosuppressive CsA analogues with anti HIV-1 activity were described to date. Representative examples such as NIM 811[6] and [(D)MeSer]^3-[(4′-OH)MeLeu]^4-CsA[8] correspond either to a modification on [MeLeu]^4 or both on [Sar]^3 and [MeLeu]^4 (Fig. 1). Other cyclosporin analogues that are active against HIV-1 replication possess either one modification on the [MeBmt]^1 or [MeLeu],[4,6] two modifications on the [MeBmt]^1 or [αAbu]^2 and [MeLeu],[4] or three modications on the [αAbu],[2] [Sar]^3 and [MeLeu]^4 residues.[9]

The present report describes the regioselective and stereoselective synthesis and the pharmacological properties of a novel series of CsA analogues **3a–o**[10,11] with the necessary *R*-absolute configuration at the sarcosine residue 3.[9,12] These new semisynthetic CsA derivatives containing one or two functional modifications of the methylene group of the [Sar]^3 residue and the 4′-hydroxylation of the [MeLeu]^4 residue were tested for their in vitro anti-HIV (HIV-1_{Lai} strain) and immunosuppressive (inhibition of IL-2 production) activity. As shown in Table 1, the most potent compounds **3k** and **3l** displayed in vitro anti-HIV activity with IC_{50}'s of ~46 nM and exhibited low immunosuppressive activity with IC_{50}'s of 1549 and 6047 nM, respectively.

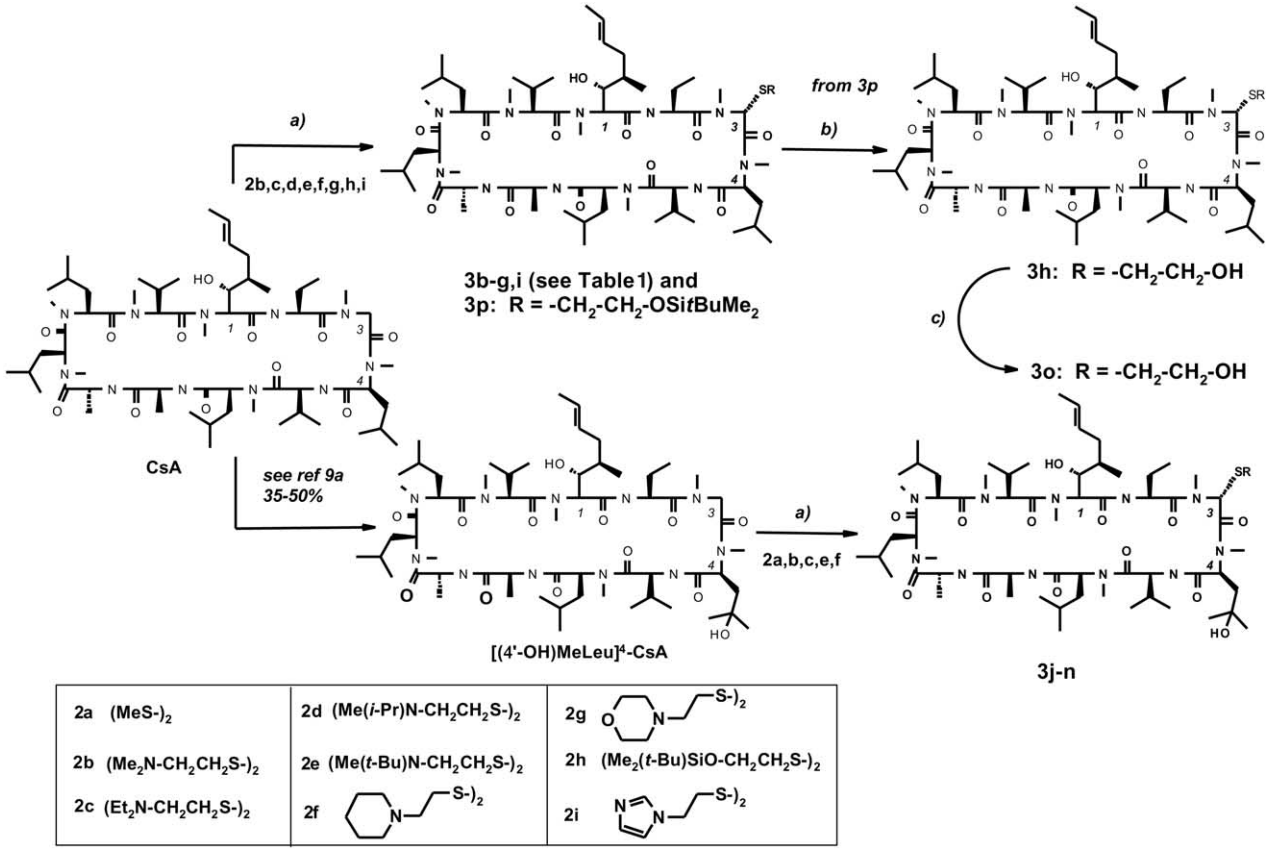
Chemistry

CsA presents two obvious sites for chemical modifications, the allylic system of the [MeBmt]^1 and the methylene group of the sarcosine residue [Sar]^3. The targeted cyclosporin A derivatives **3b–o** bearing a thioalkyl groups at position 3 were prepared in one, two or three steps starting from either commercially available CsA or [(4′-OH)MeLeu]^4-CsA (Scheme 1). The latter was synthesized by an enzymatic hydroxylation of CsA at [MeLeu]^4 by the strain *Sebekia benihana* (35% yield without recycling, 50% with one recycling step, >90% HPLC purity) according to a previous work.[9a]

To our knowledge, few C-thioalkylation of the sarcosine of CsA were described by Seebach D. and col. via the generation of the hexalithio derivative of CsA,[12] and none described the preparation of polylithio derivative of [(4′-OH)MeLeu]^4-CsA and condensation with electrophiles.

The C3-regio and stereoselective alkylthiolation of CsA and [(4′-OH)MeLeu]^4-CsA was performed using different experimental conditions, that is by the action of various dialkyl disulfides **2a–i**[13] as electrophiles with either CsA-polyanion or [(4′-OH)MeLeu]^4-CsA-polyanion intermediates which were themselves obtained, respectively, from CsA and [(4′-OH)MeLeu]^4-CsA by the action of strong bases. The relative stereochemistry of the former sarcosine residue was determined by ^{1}H NMR spectroscopy by comparison with already described analogues.[14] A difference at least 0.2 ppm high field being observed for the 3*R* isomer compared to the 3*S* one. In addition, the X-ray structure of the representative compound **3k** confirms the R conformation of the substituent on [Sar]^3.[15]

Several synthetic approaches were used, such as Method A (preparation of **3b**,**c**,**j**,**k**): addition of lithium diisopropylamide (LDA, 15–16 equiv) followed by the addition of *n*-Buli (6–7 equiv) in THF to a solution of CsA (1 equiv) or [(4′-OH)MeLeu]^4-CsA (1 equiv); Method B (preparation of **3f**): addition of CsA (1 equiv) in a mixture of 1,3 - dimethyl - 3,4,5,6 - tetrahydro - 2(1*H*) - pyrimidone and THF to LDA (15 equiv) in THF; Method C (preparation of **3d**,**e**,**g**,**i**,**m**,**n**,**p**): addition of CsA (1 equiv) or [(4′-OH)MeLeu]^4-CsA (1 equiv) in THF or *t*-BuOMe *t*-$NaNH_2$ (1–2 equiv) prepared in situ [from Na (12–18 equiv) in liq NH_3 at −33 °C]; Method D (preparation of **3l**): addition of $NaNH_2$ (16 equiv) in liq. NH_3 at −33 °C to [(4′-OH)MeLeu]^4-CsA (1 equiv) in *t*-BuOMe. The cyclosporin A derivatives **3b–g**,**i**,**j–n**,**p** were purified by flash chromatography on silica gel and/or neutral alumina with poor to moderate non-optimized yields (4–44%). Compound **3o** was obtained in two steps from **3p** (–SR = –S–CH_2–CH_2–Si–*t*$BuMe_2$) by the action of trifluoroacetic acid—giving **3h** in 69% yield—followed by an enzymatic hydroxylation at



Scheme 1. Synthesis of **3b–o**. Experimental conditions: (a) Method A (**3b**,**c**,**j**,**k**); (i) for **3b**,**c**: CsA (1 equiv) and for **3j**,**k**: [(4′-OH)MeLeu]^4-CsA (1 equiv), THF, −78 °C then LDA (15–16 equiv), THF, −78 °C, 10–20 min then *n*-BuLi (1.6 M hexane, 6–7 equiv), 0 °C, 20–30 min; (ii) **2b**: 22 equiv, **2c**: 3 equiv, **2a**: 3 equiv), −78 °C, 30 min then 0 °C, 18 h; (iii) for **3b**,**c**: H_2O, AcOH until pH = 7, for **3k**,**j**: 36% HCl until pH = 7); (iv) flash chromatography on silica gel (**3b**: MeOH–H_2O–CH_2Cl_2 18/2/84, **3c**: MeOH–CH_2Cl_2 93.5/6.5, **3k**: MeOH–CH_2Cl_2 98/2, **3j**: AcOEt–acetone 85/15); **3b**: 11%, **3c**: 11.5%, **3k**: 4%, **3j**: 13%. Method B (**3f**): (i) LDA (15 equiv), THF, −78 °C then CsA (1 equiv), 1,3-dimethyl-3,4,5,6-tetrahydro-2(1*H*)-pyrimidone, THF, −40 °C, 20 min then **2f** (14 equiv), THF, −78 °C, 10 min then rt, 12 h; (ii) H_2O, AcOEt; (iii) flash chromatography on silica gel (AcOEt), **3f**: 9%. Method C (**3d**,**e**,**g**,**i**,**n**,**m**,**p**): (i) liq NH_3, −33 °C, Na (1–2 equiv), $Fe(NO_3)_3$, $9H_2O$ (0.06–0.13 equiv) then when the aspect solution changes from a blue solution to a grey suspension, Na (12–18 equiv), −33 °C, 1–2 h; (ii) for **3d**,**e**,**g**,**i**: CsA (1 equiv) and for **3n**,**m**,**p**: [(4′-OH)MeLeu]^4-CsA (1 equiv), THF or *t*BuOMe (for **3m**) then **2d** and **2e**: 4 equiv, **2i**: 10 equiv, **2f**: 5 equiv, **2g**: 0.7 equiv, **2e**: 0.6 equiv, **2h**: 20 equiv, THF or dioxane (for **3n**) or *t*BuOMe (for **3m**), −33 °C to rt, 12–19 h; (iii) flash chromatography on silica gel: **3d**,**e**,**p**: AcOEt–MeOH 4/1, **3d**: 10%, **3e**: 5%, **3p**: 26%, **3g**,**i**: AcOEt–MeOH 19/1, **3g**: 6%, **3i**: 10%, **3m**: CH_2Cl_2–EtOH 19/1, 37%, flash chromatography on neutral alumina: **3n**: AcOEt–cyclohexane 4/1, 31%. Method D (**3l**): (i) $NaNH_2$ (17 equiv), liq NH_3, −33 °C then [(4′-OH)MeLeu]^4-CsA (1 equiv), *t*BuOMe, −33 °C, 1.5 h then **2c** (8 equiv), −33 °C, 20 min; (ii) NH_4Cl, −33 °C to rt, 2.5 h; (iii) *t*BuOMe, H_2O, $MesO_3H$ until pH = 1.5–2; (iv) 20% NH_3 until pH = 9; (v) flash chromatography on neutral alunina (AcOEt–EtOH 9/1) then flash chromatography on silica gel (MeCN–MeOH–(28%) NH_3 260/40/3), **3l**: 44%. (b) (i) 1 M TFA, THF, 20 °C, 4 h; (ii) flash chromatography on silica gel (AcOEt) then chromatography on neutral alumina (AcOEt–cyclohexane 4/1), 3 h: 69%; (c) *Sebekia benihana*, EtOH, 28 °C then chromatography on silica Sorbsil (AcOEt–cyclohexane 4/1), **3o**: 5%.

MeLeu-4 of **3h** using *S. benihana* with non-optimized poor yield (5%).

To our knowledge, no peptide or cyclopeptide such as CsA and [(4′-OH)MeLeu]^4-CsA has been treated with sodamide/liquid ammonia as a base to give the corresponding polyanion before. This therefore represents an original and useful method for the functionalization of CsA and [(4′-OH)MeLeu]^4-CsA at position 3 without excess of *n*-BuLi.[16]

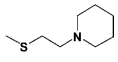
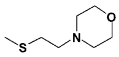
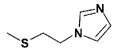
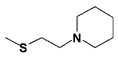
Anti-AIDS Activity

The in vitro anti-HIV activity of compounds **3a–o**, CsA, [(4′-OH)MeLeu]^4-CsA, NIM 811 and [(D)MeSer]^3-[(4′-OH)MeLeu]^4-CsA was measured in established cell line cultures.[17] Thus, the CEM4 cell line was infected with HIV-1_{Lai} strain. The inhibition of HIV replication in the culture was estimated by the measure of the reverse transcriptase (RT) produced in the supernatant. Antiviral activity was expressed as the IC_{50} RT, the concentration required to reduce replication of HIV by 50%, and was determined by linear regression (Table 1). Activity has been confirmed in primary peripheral blood mononuclear cells.

Immunosuppressive Activity

CSA is known to block the signaling pathway which leads to IL2 production upon T-cell stimulation and to inhibit T cell proliferation.[18] The immunosuppressive activity of **3a–o**, CsA, [(4′-OH)MeLeu]^4-CsA, NIM 811 and [(D)MeSer]^3-[(4′-OH)MeLeu]^4-CsA was evaluated by inhibition of the IL2 production in Jurkat cells. The concentration required to reduce IL2 production by 50% (IC_{50}) was determined by linear regression (Table 1).

Table 1. In vitro anti-HIV and immunosuppressive activities of **3a–o**, CsA, [(4′-OH)MeLeu]^4-CsA, NIM 811 and [(D)MeSer]^3-[(4′-OH)MeLeu]^4-CsA

	Compd	–SR	In vitro anti-HIV activity RT[a] (IC_{50}, nM)	Immunosuppressive activity IL2[b] (IC_{50}, nM)
CsA derivatives	**3a**[12a] (from **2a**)	–S–Me	65.5	3
	3b (from **2b**)	S–CH_2–CH_2–N(Me)$_2$	35	47
	3c (from **2c**)	–S–CH_2–CH_2–N(Et)$_2$	2.5	15
	3d (from **2d**)	–S–CH_2–CH_2–N(Me)–*i*–Pr	19.5	61
	3e (from **2e**)	–S–CH_2–CH_2–N(Me)–*t*–Bu	24	277
	3f (from **2f**)		72	97
	3g (from **2g**)		101	128
	3h (see text)	–S–CH_2–CH_2–OH	70	19
	3i (from **2i**)		80.5	165
[(4′-OH) MeLeu]^4-CsA derivatives	**3j** (from **2a**)	–S–Me	84	110
	3k (from **2b**)	–S–CH_2–CH_2–N(Me)$_2$	45	1549
	3l (from **2c**)	–S–CH_2–CH_2–N(Et)$_2$	47	6047
	3m (from **2e**)	S–CH_2–CH_2–N(Me)–*t*–Bu	43	1572
	3n (from **2f**)		19.5	882
	3o (see text)	–S–CH_2–CH_2–OH	80	959
	CsA		455	2
	[(4′-OH)MeLeu]^4-CsA		137.5	166
	NIM 811		293	934
	[(D)MeSer]^3-[(4′-OH) MeLeu]^4-CsA		95	783

[a]CEM4 infected with HIV-1_{Lai}.
[b]Human Jurkat-clone E6-1 leukemia T-cell line.

The following structure–activity relationship features were identified (Table 1). The introduction of a methylthio group at the 3-position of CsA both slightly increased the anti-HIV and strongly increased the immunosuppressive potency, 7-fold and ~340-fold, respectively (comparison of **3a** vs **CsA**). Introduction of an amino group on this chain maintained (**3f–i**) or improved the anti-HIV potency (**3b–e**) versus **3a** (2- to 40-fold), whereas the immunosuppressive activity was reduced (5.4- to 100-fold). In this series, the most potent anti-HIV compound was **3c** bearing a diethylaminoethylthio group at the 3-position, exhibiting an IC_{50} of 2.5 nM but also an excellent immunosuppressive activity with an IC_{50} of 15.3 nM.

Following previous work showing that **CsA** derivatives with substituents at positions 1, 4, 6 and/or 11 displayed reduced immunosuppressive activity, we decided to take [(4′-OH)MeLeu]^4-CsA as starting point and to prepare non-immunosuppressive anti-HIV CsA derivatives. [(4′-OH)MeLeu]^4-CsA demonstrated both moderate anti-HIV and immunosuppressive potency (IC_{50} = ~140–150 nM). The introduction of a hydroxyl group at the 4′-position of [Me-Leu]^4 of CsA reduced slightly the anti-HIV activity (6-fold vs CsA) and strongly reduced the immunosuppressive activity (83-fold vs CsA). The combined introduction of a hydroxyl group at the 4′-position of [Me-Leu]^4 and an alkylthio group on [Sar]^3 increased the anti-HIV activity (1.6- to 7-fold) and significantly deceased the immunosuppressive activity (5- to 36-fold, **3k–o** vs [(4′-OH)MeLeu]^4-CsA) except for **3j** showing a similar anti-HIV and immunosuppressive activity to the parent compound [(4′-OH)MeLeu]^4-CsA. Note that **3j** has no polar function such as an amino or a hydroxy group in β-position to the sulfur atom link to the [Sar]^3. Compared with **3b–f** and **3h**, the introduction of a hydroxyl group at the 4′-position of [Me-Leu]^4 either maintained (**3k** vs **3b**, **3o** vs **3h**), reduced (**3l** vs **3c**, **3m** vs **3e**) or increased (**3n** vs **3f**) the anti-HIV activity. The most potent CsA derivatives in the present series were **3k** and **3l** demonstrating potent anti-HIV-1 activity (IC_{50} ~46 nM) and low immunosuppressive activity (IC_{50} = 1549 and 6047 nM, respectively), and being 80- to 3000-fold less immunosuppressive than CsA.

Compounds **3k** was studied for its ability to compete with CsA binding to cyclophilin A. **3k** displayed CsA binding to cyclophilin A with IC_{50}'s of 118 nM, and was about 3-fold more potent than CsA itself (IC_{50} = 352 nM). Like CsA, **3k** and **3l** did not inhibit the HIV-2 strain $_{Rod}$ isolate which is restricted to Western Africa

($IC_{50} > 10000$ nM). Finally, **3k** and **3l** were freely soluble in water (≥ 100 mg/mL) under their methanesulfonate forms compatible with oral administration. Compared with NIM 811 and [(D)MeSer]^3-[(4′-OH)MeLeu]^4-CsA, 3k and 3l exhibited 2- and 6-fold higher anti-HIV potency and 1.6- to 6- and 2- to 8-fold lower immunosuppressive activities, respectively.

In conclusion, this study reports novel semisynthetic CsA derivatives devoided of immunosuppressive effects with potent in vitro anti-HIV activity (inhibition of HIV-1 replication). The most active compounds **3k** and **3l** were synthesized in two steps starting from commercially available CsA. **3k** and **3l** showed strong anti-HIV-1 activity (IC_{50} ~46 nM) and low immunosuppressive potency ($IC_{50} \geq 1500$ nM). They represent original and potential candidates for the treatment of HIV-1 infection and could be efficiently combined with other anti HIV drugs.

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11. All new compounds exhibited spectral (^{1}H NMR, ^{13}C NMR, IR) and analytical (elemental analysis and/or MS) data fully consistent with the assigned structures. **3b**: white solid (mp 155 °C), **3c**: white solid (mp 140 °C), **3d**: white solid (mp 70 °C), **3e**: white solid (mp 130 °C), **3f**: pale yellow solid (mp 132 °C), **3g**: pale yellow solid (mp 154 °C), **3h**: white solid (mp 139 °C, **3i**: yellow solid (mp 208 °C), **3j**: white solid (mp 140 °C), **3k**: white solid (mp 140 °C), **3l**: white solid (mp 140 °C), **3m**: white solid (mp 126–136 °C), **3n**: yellow solid (mp 143 °C), **3o**: white lac (mp 45 °C), **3p**: pale yellow solid (mp 125 °C).
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18. Cells were cultured in the presence of serial dilutions of tested compound or solvent alone and stimulated with a mixture of the two mitogens, PHA and PMA. After 24 h of incubation, the level of IL-2 in the culture supernatant was measured with an ELISA kit. IL-2 concentrations were calculated and the IC_{50} (concentration required to reduce IL-2 production by 50%) was determined by linear regression.