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Short communication

New 7-hydroxy-1,3-diazabicyclo[3.3.0]octane derivatives: evaluation of their in vitro immunomodulating effects

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Summary — In order to improve the water solubility of some previously reported immunoactive dioxothiadiazabicyclo[3.3.0]octanes, we synthesized a series of new diazabicyclo[3.3.0]octanols from the *trans*-4-hydroxy-L-proline methyl ester in two steps. Acylation of the ester with an isocyanate or an isothiocyanate under the appropriate conditions afforded *N*-acylated derivatives exclusively. Then through a cyclization process in the presence of sodium methylate, bicyclic derivatives were obtained, most of them as a mixture of two diastereomers which were separated by column chromatography. A mitogenic stimulation assay using the T-cell mitogen phytohemagglutinin was performed with human peripheral blood leukocytes in the presence of the different synthesized compounds and with levamisole as reference. Several compounds showed marked stimulant effects on the proliferation of lymphocytes as compared to levamisole, but no correlation could be established between molecular configuration and stimulation or inhibition effects on proliferation.

diazabicyclo[3.3.0]octane derivative / nuclear Overhauser effect / immunomodulatory agent / T lymphocyte / proliferation assay

Introduction

Immunostimulating drugs are considered to offer therapeutic possibilities as a useful complement in the chemotherapy of cancer and diseases related to an immunodeficiency. Most of the substances used today in clinical practice to enhance the immunoresponse are bacterial or biochemical preparations. However, since the first observation by Renoux in 1971 [1] reporting that anthelmintic levamisole was able to increase the immune response in laboratory animals, various synthetic substances structurally related or not to levamisole have been prepared and their immunological profile investigated [2–7].

We have focused our interest on the synthesis and biological evaluation of azabicyclic compounds including in their structure a bridgehead nitrogen atom [8–11]. We have reported that most of these present significant activity in several biological tests: phytohemagglutinin-induced lymphocyte T proliferation, soluble interleukine-2 receptor release, and oxidative response of mice peritoneal macrophages. But the tested compounds were sparingly soluble in aqueous medium, so that their in vitro evaluation was sometimes difficult.

In the present paper we report the synthesis and evaluation on human lymphocyte proliferation of a new series of diazabicyclo[3.3.0]octanols including in their structure the hydroxy group as a hydrophilic moiety. The easily available *trans*-4-hydroxy-L-proline was selected as a starting material, as it is commonly used in the synthesis of various azabicyclic compounds [12–14] as chiral compounds.

Chemistry

The dioxodiazabicyclo[3.3.0]octanols **9–13** and their sulfur-containing analogs **14** and **15** were prepared in three steps from the natural amino-acid *trans*-4-hydroxy-L-proline, the absolute configuration of which is 2*S*-4*R* (scheme 1). First its methyl ester **1** was obtained in good yield as an hydrochloride, by reacting at low temperature the amino acid with an excess of methanol and thionyl chloride [15]. The reaction of **1** with an equivalent of isocyanate or isothiocyanate at 0 °C afforded the *N*-substituted deriva-

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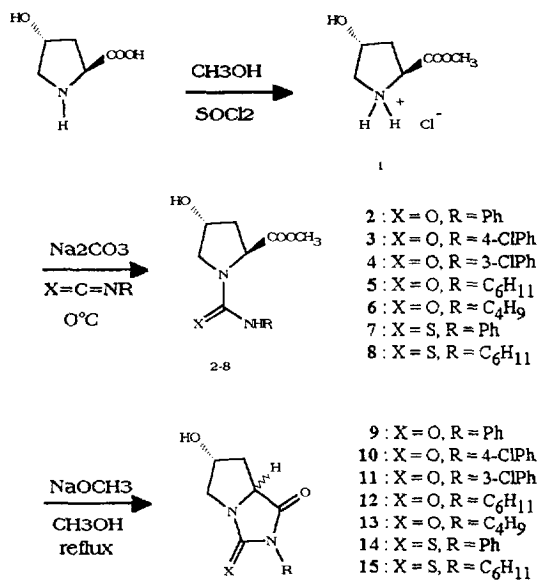


Scheme 1. Synthetic route to 7-hydroxy-1,3-diazabicyclo-[3.3.0]octanes.

tives **2–8** without preliminary protection of the OH functional group, which remained unaffected. Physical constants and NMR spectral data for these derivatives are summarized in tables I and II. In the infrared spectra, two strong absorptions can be observed near 1735 cm^{-1} for the ester group and at 1610–1640 or 1340 cm^{-1} for the amidic or thioamidic groups, respectively. When the reaction was performed with an excess of isocyanate or isothiocyanate at room temperature, a mixture of *O*- and *N*-substituted derivatives was obtained.

The cyclization of compounds **2–8** was performed by refluxing with sodium methylate in absolute methanol. Sodium was eliminated by treatment with a cation exchange resin. Bicyclic derivatives **13–15** were obtained as a single isomer, and compounds **9–12** as a mixture of two diastereomers which were separated by column chromatography.

The first step of the reaction leads to the formation of a lactim sodium salt:

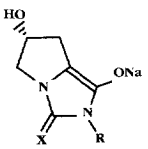
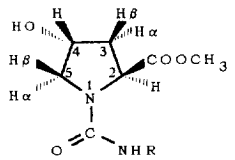


Table I. Physical constants of compounds **2–15**.

Compound	*Yield (%)*	*Mp(°C)*	$[\alpha]_D^{20a}$	*Formula*	*Analysis*	*MS* $M^{+\cdot}$
2	70	171	−70 (5.8)	$C_{13}H_{16}N_2O_4$	(C, H, N)	
3	66	188	−68 (2.3)	$C_{13}H_{15}ClN_2O_4$	(C, H, N, Cl)	
4	67	194	−75 (4.2)	$C_{13}H_{15}ClN_2O_4$	(C, H, N, Cl)	
5	66	147	−49 (3.6)	$C_{13}H_{22}N_2O_4$	(C, H, N)	
6	85	118	−56 (4.2)	$C_{11}H_{20}N_2O_4$	(C, H, N)	
7	85	148	+38 (4.9)	$C_{13}H_{13}N_2O_3S$	(C, H, N, S)	
8	72	177	+5 (3.7)	$C_{13}H_{22}N_2O_3S$	(C, H, N, S)	
9	83	a: 155 b: 165	+64 (2.3) −32 (2.3)	$C_{12}H_{12}N_2O_3$	(C, H, N)	232
10	71	a: 181 b: 187	+53 (2.2) −36 (2.4)	$C_{12}H_{11}ClN_2O_3$	(C, H, N, Cl)	266
11	73	a: 125 b: 165	+80 (2.5) −111 (2.1)	$C_{12}H_{11}ClN_2O_3$	(C, H, N, Cl)	266
12	72	a: 144 b: 186	+35 (3.2) −43 (1.8)	$C_{12}H_{18}N_2O_3$	(C, H, N)	238
13	73	130	+5 (1.6)	$C_{10}H_{13}N_2O_3$	(C, H, N)	212
14	85	146	+186 (2.9)	$C_{12}H_{12}N_2O_3S$	(C, H, N)	248
15	80	105	+12 (1.4)	$C_{12}H_{18}N_2O_2S$	(C, H, N, S)	254

[a]In DMSO (%).

Table II. Spectral data for *N*-substituted 4-hydroxy-2-methoxycarbonyl-1-carbamoylpyrrolidines and their sulfur analogs **2–8**.



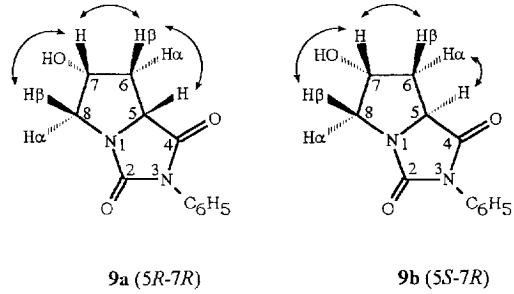
Compound	*^{1}H-NMR (DMSO-d_6) (δ, ppm)*
2	1.95 (1H, ddd, J = 13, 8, 5 Hz, H3α), 2.15 (1H, ddd, J = 13, 8, 3 Hz, H3β), 3.45 (1H, d, J = 11 Hz, H5α), 3.64 (3H, s, OCH_3), 3.68 (1H, dd, J = 11, 5 Hz, H5β), 4.4 (1H, m, H4), 4.44 (1H, br d, J = 8 Hz, H2), 5.25 (1H, s, OH), 7–7.5 (5H, m, Ph), 8.4 (1H, s, NH)
3	1.95 (1H, ddd, J = 13, 8, 5 Hz, H3α), 2.16 (1H, ddd, J = 13, 8, 3 Hz, H3β), 3.43 (1H, d, J = 11 Hz, H5α), 3.64 (3H, s, OCH_3), 3.67 (1H, dd, J = 11, 5 Hz, H5β), 4.4 (1H, m, H4), 4.44 (1H, br d, J = 8 Hz, H2), 5.22 (1H, s, OH), 7.3 (2H, dd, J = 8 Hz, R), 7.6 (2H, dd, J = 8 Hz, R), 8.6 (1H, s, NH)
4	1.95 (1H, ddd, J = 13, 8, 5 Hz, H3α), 2.17 (1H, ddd, J = 13, 8, 3 Hz, H3β), 3.47 (1H, d, J = 11 Hz, H5α), 3.66 (3H, s, OCH_3), 3.67 (1H, m, H5β), 4.4 (1H, m, H4), 4.46 (1H, br d, J = 8 Hz, H2), 5.26 (1H, s, OH), 7.0–7.5 (3H, m, R), 7.7 (1H, s, R), 8.6 (1H, s, NH)
5	1.1–1.7 (10H, m, $5CH_2$ cyclohexyl), 1.85 (1H, ddd, J = 13, 8, 5 Hz, H3α), 2.05 (1H, ddd, J = 13, 8, 4 Hz, H3β), 3.2 (1H, dd, J = 10, 2 Hz, H5α), 3.35 (1H, m, CH cyclohexyl), 3.47 (1H, dd, J = 10, 5 Hz, H5β), 3.7 (3H, s, OCH_3), 4.27 (1H, t, J = 8 Hz, H4), 4.32 (1H, dd, J = 8, 4 Hz, H2), 5.11 (1H, s, OH), 6.0 (1H, s, NH)
6	0.9 (3H, t, J = 7 Hz, CH_3), 1.25 (2H, sext, J = 13, 7 Hz, CH_2), 1.35 (2H, qt, J = 13, 7 Hz, CH_2), 1.85 (1H, ddd, J = 13, 7, 5 Hz, H3α), 2.06 (1H, ddd, J = 13, 8, 4 Hz, H3β), 2.95 (2H, m, CH_2), 3.2 (1H, dd, J = 10, 2 Hz, H5α), 3.45 (1H, dd, J = 10, 5 Hz, H5β), 3.65 (3H, s, OCH_3), 4.28 (1H, t, J = 8 Hz, H4), 4.35 (1H, dd, J = 7, 4 Hz, H2), 5.2 (1H, s, OH), 6.25 (1H,s, NH)
7	2.05 (1H, m, H3α), 2.22 (1H, ddd, J = 13, 8, 4 Hz, H3β), 3.6 (1H, d, J = 10 Hz, H5α), 3.65 (3H, s, OCH_3), 3.8 (1H, dd, J = 10, 5 Hz, H5β), 4.49 (1H, m, H4), 4.9 (1H, m, H2), 5.35 (1H, s, OH), 7.1-7.4 (5H, m, Ph), 9.3 (1H, s, NH)
8	1.1–1.85 (10H, m, $5CH_2$ cyclohexyl), 1.95 (1H, m, H3α), 2.1 (1H, ddd, J = 13, 8,5 Hz, H3β), 3.4 (1H, br d, J = 11 Hz, H5α), 3.6 (3H, s, OCH_3), 3.63 (1H, m, H5β), 4.08 (1H, br q, J = 7.5, 3.5 Hz, CH cyclohexyl), 4.4 (1H, m, H4), 4.78 (1H, m, H2), 5.26 (1H, s, OH), 7.1 (1H, s, NH)

When the sodium is removed, the unstable lactim group is changed into a lactam group, so that the enolic proton can be transferred to either the 5*R*- or 5*S*- position. Therefore the resulting diastereomers were either 5*R*-7*R* or 5*S*-7*R*.

Physical constants and NMR spectral data for these derivatives are summarized in tables I and III. Infrared spectra for compounds **9–13** show a broad absorption near 1650 cm^{-1} attributable to both carbonyl groups; in the case of the thia analogs **14** and **15**, two bands can be observed at 1700 and 1340 cm^{-1}, attributable to the carbonyl and thiocarbonyl groups respectively.

^{1}H NMR spectra of all compounds were performed at high field (400 MHz). On account of the complexity of these spectra, we have chosen to realize a more complete study on compounds **9a** and **9b**. For each of these diastereomers, the different coupling constants were determined by enlarging spectra, and their configurations by nuclear Overhauser effect (NOE) experiments (fig 1).

For **9a**, NOEs were observed between the H6β proton signal at δ 2.5 and H7 at δ 4.5 and H5 at δ 4.6 ppm, showing that these protons are in a *cis* relationship to one another. For **9b**, NOEs were observed between the H6α proton signal at δ 2.3 and H5 at δ 4.5, and between the H6β proton signal at δ 2.4 and H7 at δ 4.6 ppm, showing that H6α and H5, and H6β and H7, are in a *cis* relationship to one another. The enlarged proton NMR spectra of **9a** and **9b** allowed analysis of the six coupling proton system according to



9a (5*R*-7*R*) **9b** (5*S*-7*R*)

Fig 1. Configuration of the two isomers **9a**, **9b** and NOEs.

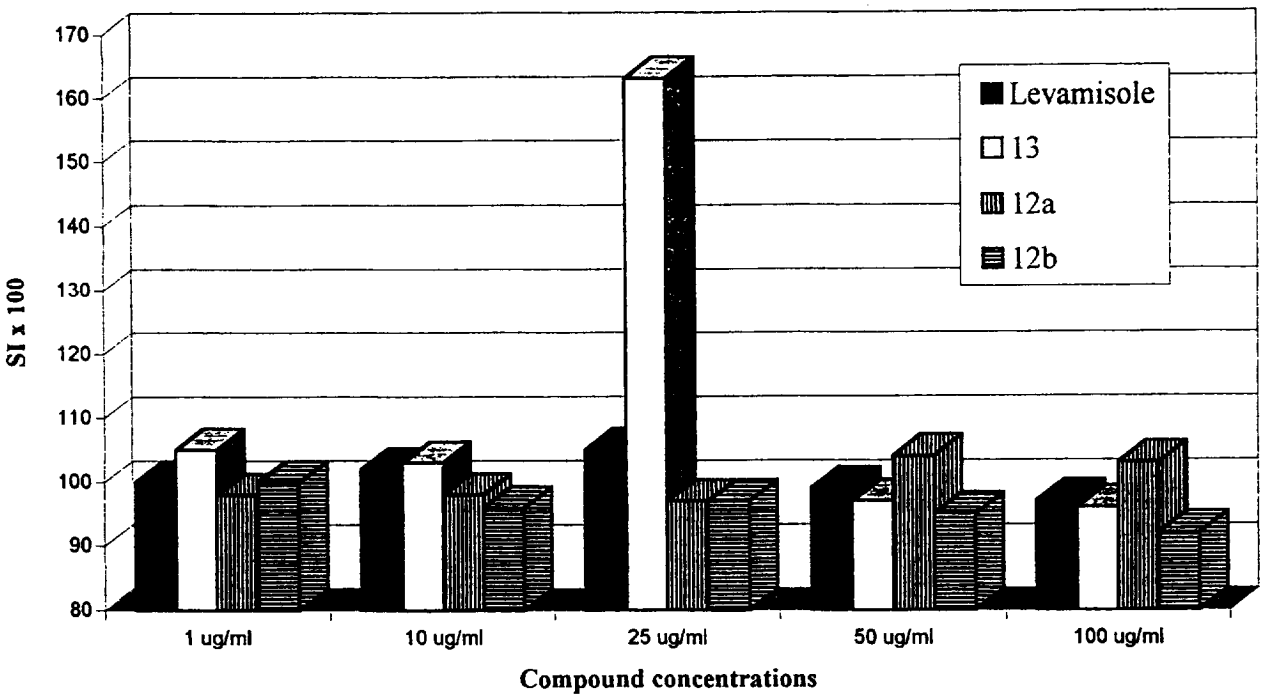


Fig 2. Median of stimulation index (SI) of human lymphocytes stimulated in vitro by phytohemagglutin (PHA, 25 μg/mL) obtained at increasing concentrations of levamisole (n = 11) and compounds **12a**, **12b** and **13** (n = 5).

the Abraham–McLaughlan procedure [16]. NOEs were confirmed by coupling constants between H5, H6α, H6β, H7, H8α and H8β protons (see the *Experimental protocols*). The configurations of the two diastereomers are 5*R*-7*R* for the major product and 5*S*-7*R* for the minor one.

Biological results and discussion

A mitogenic stimulation assay using the T-cell mitogen phytohemagglutinin (PHA) was performed with peripheral blood leukocytes taken from healthy donors. Proliferative cells were evaluated using a quantitative colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). This colorimetric assay converts yellow tetrazolium salt to the blue formazan derivative via the mitochondrial enzymes in viable cells [17].

Assays for several compounds were performed on leukocytes from five donors, and those for levamisole from eleven donors. The responses to PHA are shown in figures 2 and 3. The horizontal line representing proliferation of 100%, indicates the absence of an effect by compounds on the proliferative response. Below this line, this effect indicates immunosuppressive activity and above, immunostimulant activity. The proliferative activity of levamisole, used as the reference [18], was maximum at concentrations of 1 and 10 μg/mL, and then rapidly decreased. Several compounds had significant positive effects on the proliferation of lymphocytes as compared to those of levamisole: **9a** and **9b** at 10, 25, 50 and 100 μg/mL (fig 3); **11a** at 1 and 10 μg/mL and **11b** at 10 (p = 0.05), 25, 50 and 100 μg/mL (fig 3); **12a** at 50 and 100 μg/mL and **13** at 25 μg/mL (p = 0.02) (fig 2). In contrast, at whatever the concentration used, compounds **10a**, **10b**, **12b** and **14** exhibited no significant proliferative effects. None of the compound activities was dose-dependent.

Considering these preliminary results, no significant correlation between molecular configuration and stimulation or inhibition effects on proliferation could be established.

The presence of an alkyl group such as n-butyl on the bicyclic system seems to enhance activity between 1 and 25 μg/mL. For aromatic substituents, chlorine in para position seems favorable for activity; however **11a** is better at the lowest concentrations and **11b** at the highest ones. Substitution of oxygen by sulfur leads to a negative effect, as shown for compound **14** compared to **9**.

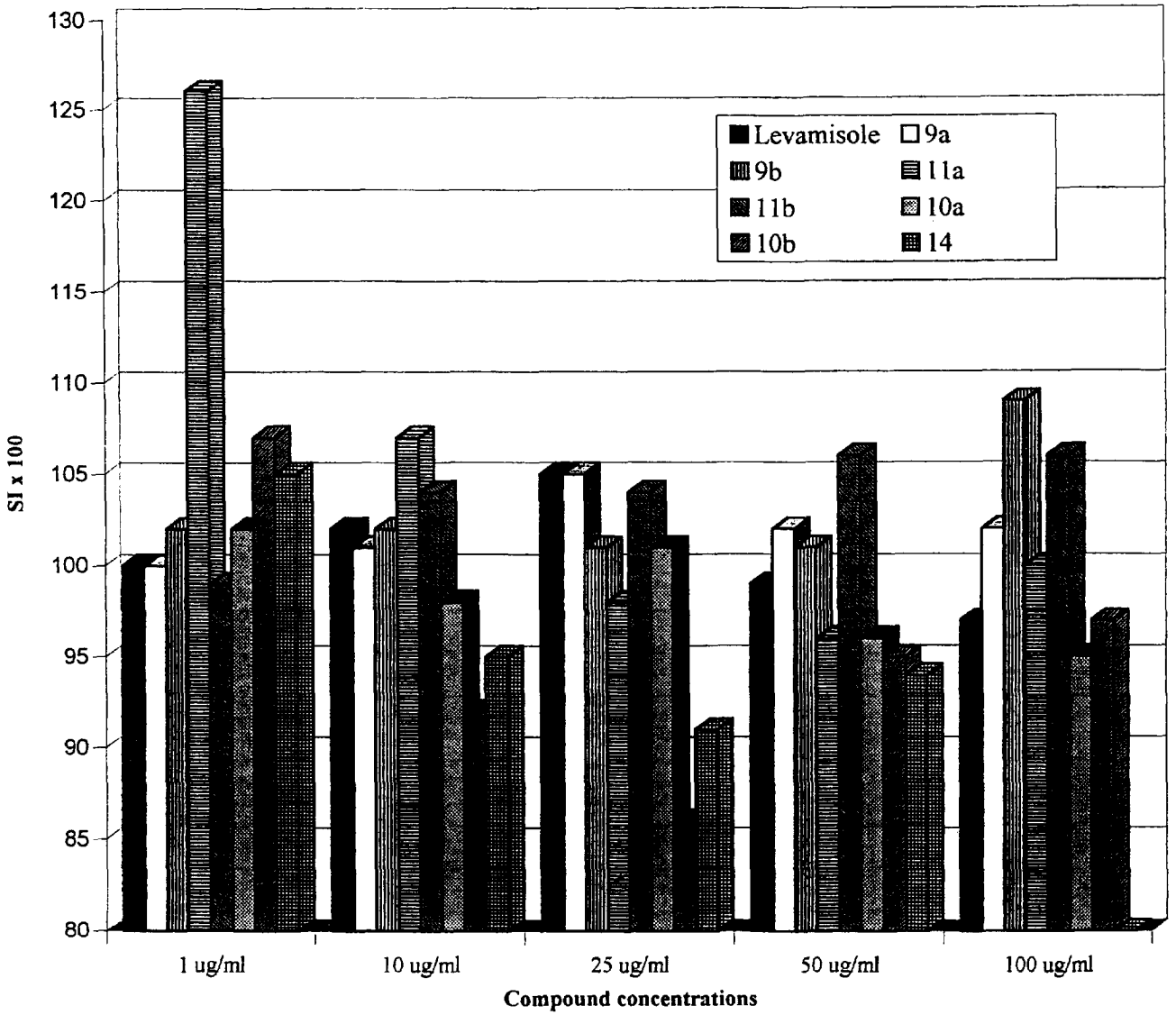


Fig 3. Median of stimulation index (SI) of human lymphocytes stimulated in vitro by phytohemagglutinin (PHA, 25 μg/mL) obtained at increasing concentrations of levamisole (n = 11) and compounds **9a,b**, **11a**, **10a,b** and **14** (n = 5).

Experimental protocols

Chemistry

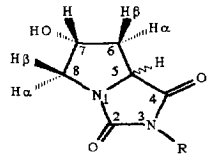
Melting points were determined on a Kofler apparatus without correction. Infrared (IR) spectra were recorded on a Beckman 4240 spectrophotometer in KBr disks. All ^{1}H and ^{13}C spectra were recorded on a Bruker 400 MSL spectrophotometer using tetramethylsilane as an internal standard. The following abbreviations are used: s = singlet, d = doublet, t = triplet, q = quartet, qt = quint, sext = sextet, m = multiplet, br = broad. Mass spectra were recorded on a Hewlett Packard 5985B apparatus. Optical rotations were measured on a Perkin-Elmer Model 141 polarimeter in dimethylsulfoxide, or in water for the hydrochloride. Analyses indicated by the symbols of the elements were within ±0.4% of theoretical values. For analytical TLC, plastic sheets coated with a 0.2 mm layer of silica gel 60 F_{254} (Merck) were used. When required, the separation of crude reaction products was achieved by chromatography on a silica gel column (70–230 mesh). The cation exchange resin (20–50 mesh) was purchased from Aldrich Chemical Company.

Trans-4-hydroxy-L-proline was purchased from Jansen Chimica, mp 274–275 °C, $[\alpha]^{20}$ –55° (H_2O), M = 131.13.

(2S,4R)-4-Hydroxy-2-methoxycarbonylpyrrolidine hydrochloride **1**

A suspension of 30 g (0.229 mol) *trans*-4-hydroxy-L-proline in 250 mL anhydrous methanol was cooled to between 0 and 5 °C, then 54.5 g (0.458 mol) thionyl chloride was added drop-

Table III. Spectral data for 3-substituted 2,4-dioxo-7-hydroxy-1,3-diazabicyclo[3.3.0]octanes and their 2-thia analogs **9–15**.



Compound	*^{1}H-NMR (DMSO-d_6) (δ, ppm)*
9a	2.3 (1H, br d, J = 13 Hz, H6α), 2.5 (1H, ddd, J = 13, 9, 5 Hz, H6β), 3.6 (1H, dd, J = 11, 3 Hz, H8α), 3.8 (1H, dd, J = 11, 5 Hz, H8β), 4.5 (1H, m, H7), 4.6 (1H, dd, J = 9, 3 Hz, H5), 5.0 (1H, s, OH), 7.0–7.6 (5H, m, Ph)
9b	2.3 (1H, ddd, J = 13, 8, 4 Hz, H6α), 2.4 (1H, ddd, J = 13, 7, 5 Hz, H6β), 3.7 (1H, dd, J = 11, 2 Hz, H8α), 3.8 (1H, dd, J = 11, 5 Hz, H8β), 4.5 (1H, br t, J = 8 Hz, H5), 4.6 (1H, m, H7), 4.7 (1H, s, OH), 6.6–7.1 (5H, m, Ph)
10a	2–2.1 (2H, m, 2H6), 3.3–3.5 (2H, m, 2H8), 4.2–4.3 (2H, m, H5, H7), 7.3 (2H, dd, J = 8 Hz, R), 7.5 (2H, dd, J = 8 Hz, R), 9.5 (1H, br s, OH)
10b	1.9–2 (2H, m, 2H6), 3.3–3.5 (2H, m, 2H8), 4.1–4.25 (2H, m, H5, H7), 4.0 (1H, βr s, OH), 7.2 (2H, dd, J = 8 Hz, R), 7.4 (2H, dd, J = 8 Hz, R)
11a	2–2.3 (2H, m, 2H6), 3.4–3.6 (2H, m, 2H8), 4.3 (1H, m, H7), 4.35 (1H, dd, J = 9, 4 Hz, H5), 7.0–7.5 (3H, m, R), 7.7 (1H, s, R), 8.5 (1H, br s, OH)
11b	1.9–2.3 (2H, m, 2H6), 3.4–3.45 (2H, m, 2H8), 4.15–4.25 (2H, m, H5, H7), 7.0-7.4 (3H, m, R), 7.6 (1H, s, R), 9 (1H, br s, OH)
12a	1.2–1.7 (10H, m, 5CH_2 cyclohexyl), 1.9 (1H, m, H6α), 2.0 (1H, ddd, J = 13, 8, 5 Hz, H6β), 3.2 (1H, d, J = 10 Hz, H8α), 3.4 (1H, dd, J = 10, 5 Hz, H8β), 3.4 (1H, m, CH cyclohexyl), 4.1–4.17 (2H, m, H5, H7), 6.3 (1H, br s, OH)
12b	1.2–1.7 (10H, m, 5 CH_2 cyclohexyl), 1.85 (1H, br dd, J = 13, 8 Hz, H6α), 2.2 (1H, m, H6β), 3.2–3.3 (2H, m, 2H8), 3.4 (1H, m, CH cyclohexyl), 3.9–4.2 (2H, m, H5, H7), 7.5 (1H, br s, OH)
13	0.9 (3H, t, J = 7 Hz,CH_3), 1.3 (2H, sext, J = 12, 7 Hz, CH_2), 1.4 (2H, qt, J = 12, 7 Hz, CH_2), 1.9 (1H, br d, J = 13 Hz, H6α), 2.0 (1H, ddd, J = 13, 8, 5 Hz, H6β), 3.0 (2H, q, J = 12, 7 Hz, CH_2), 3.2 (1H, dd, J = 11, 2 Hz, H8α), 3.31 (1H, dd, J = 11, 5 Hz, H8β), 3.95–4.11 (2H, m, H5, H7), 6.5 (1H, βr s, OH)
14	2.2–2.3 (2H, m, 2H6), 3.6 (1H,m, H8α), 3.8 (1H, dd, J = 10, 5 Hz, H8β), 4.2–4.4 (2H, m, H5, H7), 7.0–7.5 (5H, m, Ph), 11.5 (1H, s, OH),
15	1.2–1.9 (10H, m, 5 CH_2 cyclohexyl), 2.0 (1H, m, H6α), 2.1 (1H, ddd, J = 13, 8, 5 Hz, H6β), 3.4 (1H, m, H8α), 3.65 (1H, br d, J = 11 Hz, H8β), 4.0 (1H, br d, J = 8 Hz, CH cyclohexyl), 4.4–4.7 (2H, m, H5, H7), 5.2 (1H, br s, OH)

wise while stirring. At the end of the addition, the reaction mixture was kept for 4 h at room temperature. The solvent was removed under vacuo; the crude solid residue was recrystallized from a mixture of ethanol/ether (50:50) to afford 40 g (96%) of **1** as a white powder; mp 171 °C, lit 171–172 °C [15], $[\alpha]_D^{25}$ –25° (c = 4.5%, H_2O). IR (KBr): ν 3360, 1735 cm^{-1}. ^{1}H-NMR (DMSO-d_6): δ 2.1 (1H, ddd, J = 13, 11 and 4.5 Hz, H3α), 2.2 (1H, br dd, J = 13 and 7 Hz, H3β), 3.1 (1H, br d, J = 12 Hz, H5α), 3.4 (1H, dd, J = 12 and 5 Hz, H5β), 3.8 (3H, s, OCH_3), 4.4 (1H, m, H4), 4.5 (1H, dd, J = 11 and 7 Hz, H2), 5.6 (1H, br s, OH), 10.0 (2H, m, NH_2^+). ^{13}C-NMR (DMSO-d_6): δ 37.0 (C-3), 52.9 (C-5), 53.0 (OCH_3), 57.4 (C-2), 68.3 (C-4), 168.9 (C=O). Anal $C_6H_{11}NO_3$·HCl (C, H, N, Cl).

(2S,4R)-4-Hydroxy-2-methoxycarbonyl-1-phenylcarbamoyl-pyrrolidine **2**

To a solution of **1** (5 g; 0.0275 mol) in a small amount of water were added 100 mL chloroform then 30 g sodium carbonate.

The mixture was stirred at room temperature for 10 min, then filtered on a Büchner funnel and evaporated to give the base as an oil (90%). To a solution of this base in 100 mL methylene chloride at 0 °C a stoichiometric amount of phenylisocyanate (3.27 g) was added dropwise. The mixture was stirred at room temperature for 4 h, then evaporated to give a crystallized residue which was washed with ether.

Compounds **3–8** were prepared in the same manner as described for **2**. For compounds **7** and **8**, isothiocyanates were used instead of isocyanates. Physical constants and spectral data are listed in tables I and II.

(5R,7R)-2,4-Dioxo-7-hydroxy-3-phenyl-1,3-diazabicyclo-[3.3.0]octane ***9a*** *and (5S,7R)-2,4-dioxo-7-hydroxy-3-phenyl-1,3-diazabicyclo[3.3.0]octane* ***9b***

A solution of sodium methylate was prepared by dissolving sodium (0.5 g, 0.022 at g) in 20 mL anhydrous methanol, then compound **2** (2.64 g, 0.01 mol) was added. The mixture was stirred under reflux for 6 h. After partial evaporation, the residue was treated with cation exchange resin. Diastereoisomers **9a** and **9b** were separated by column chromatography with ethyl acetate/methanol (9:1) as eluent.

Other compounds **10–15** were prepared in the same manner as described for **9** but the chromatography was performed with ethyl acetate as eluent for **14** and **15**. Physical constants and spectral data are listed in tables I and III.

Biology

Proliferative responses to mitogenic stimulation (PHA)

Peripheral blood leukocytes were separated by centrifugation on a ficoll-hypaque gradient. Cells were cultured in RPMI 1640 (Gibco BRL, Cergy Pontoise, France) containing 10% fetal calf serum (DAP, Volgebrun, France) and 2 mM L-glutamine (Eurobio, Les Ulis, France). The cell suspension was diluted to 10^6/mL, and 100 µL put into each well of a 96-well plate. PHA was used at final concentration of 25 µg/mL. These incubations were carried out with and without potential immunomodulating compounds at various concentrations (1, 10, 25, 50 and 100 µg/mL), for 48 h. All cells were counted using the blue trypan (0.25%) exclusion test, and viability was always over 95%. MTT (Sigma, Saint Quentin, France) was dissolved in phosphate buffer saline at 5 mg/mL and sterilized by filtering. After the 48 h incubation, stock MTT solution (100 µL) was added to all the wells, and plates were incubated at 37 °C for 3 h. The plates were then centrifuged and dimethylsulfoxide was added to all wells. After a few minutes at room temperature, the plates were read with a Titertek Multiskan Plus (a microplate reader) using a test wavelength of 570 nm, and a reference wavelength of 630 nm. Results, calculated from triplicates, were expressed as a stimulation index (SI) using the following formula: SI = OD (T-cells stimulated by PHA + compound)/OD (T-cells stimulated by PHA). Because of the small sample size, results were expressed as median and extreme values. Statistical comparisons were made using the non-parametric Mann and Whitney *U* test [19].

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