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N-{*trans*-2-[[2′-(Acetylamino)cyclohexyl]oxy]acetyl}-L-alanyl-D-glutamic Acid: A Novel Immunologically Active Carbocyclic Muramyl Dipeptide Analogue

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A novel non-pyrogenic carbocyclic muramyl dipeptide (MDP) analogue, *N*-{*trans*-2-[[2′-(acetylamino)cyclohexyl]oxy]acetyl}-L-alanyl-D-glutamic acid, was obtained by replacement of the *N*-acetylmuramic acid part and the D-isoglutamine residue of the MDP molecule by a *trans*-2-[[2′-(acetylamino)cyclohexyl]oxy]acetyl moiety and D-glutamic acid, respectively. The title compound was selected as a promising candidate for further evaluation among several related analogues on the basis of an immunorestoration test in mice. This novel *nor*-MDP analogue protects mice against the immunosuppressive effect of cyclophosphamide and increases the nonspecific resistance of mice against fungal infection. It is an immunomodulator which enhances the maturation of lymphocytes B to plasma cells and increases the activity of lymphocytes B and lymphocytes T as well as that of macrophages but does not alter the number of these cells.

Introduction

N-(Acetylmuramyl)-L-alanyl-D-isoglutamine (muramyl dipeptide, MDP, **1**; Chart 1) was identified in 1974 as the minimal immunologically active component of bacterial cell wall peptidoglycan.[1,2] The main biological activities of MDP and its derivatives are (a) adjuvant activity, i.e., stimulation of antibody production when injected with an antigen and induction of delayed-type hypersensitivity; (b) stimulation of nonspecific resistance against bacterial, viral, and parasitic infections and against tumors; and (c) somnogenic activity.[3−8] MDP also induces other, mostly undesirable, toxic responses, such as pyrogenicity, induction of autoimmune response, and inflammatory reactions.[7−9] The immunomodulating activity of MDP is based preferentially on stimulation of macrophages as well as on T and B lymphocyte functions.[3−6,8,10−13] Many research groups have been concerned with the synthesis and immunological studies of derivatives of this highly active glycopeptide in order to obtain molecules with improved and more defined pharmacological profiles.[3,4] These efforts resulted in the recent introduction of N^2-[*N*-(acetylmuramyl)-L-alanyl-D-isoglutaminyl]-N^6-stearoyl-L-lysine (romurtide, **2**) for the treatment of leukopenia due to radiotherapy.[14,15] Whereas studies of the structure−activity relationships for this class of compounds have revealed the importance of the dipeptide moiety for immunomodulatory activity, it was found that the intact *N*-acetyl-D-glucosamine moiety is not essential for immunostimulant activity of MDP and analogues.[3,4,16] Essential prerequisites for immunostimulatory activity are *R*-absolute configuration of the first amino acid and *S*-absolute configuration of the C-terminal amino acid of the dipeptide moiety, whereas it has been shown by numerous examples that the sugar moiety can be derivatized or replaced with simple acyl groups without a significant change of immunological activity as exemplified by immunologically active lipophilic 6-*O*-acyl-MDP derivatives,[17−20] FK-156,[21−24] pimelautide,[25,26] 7-(oxoacyl)-L-alanyl-D-isoglutamines,[27] *N*-[2-(2-aminoalkoxy)propanoyl]-L-alanyl-D-isoglutamine derivatives,[28] and carbocyclic MDP analogues in which the polyhydroxy-substituted pyranose ring of D-glucosamine in MDP was replaced by a more lipophilic cyclohexane ring.[29−32] Omission of a methyl group in the lactoyl moiety of MDP produced *nor*-MDP which is less active than MDP but is also less toxic.[3] With replacement of the α-carboxamido group in the D-isoglutamine residue of MDP by a carboxyl group, the adjuvant and antiinfectious activities remain almost unchanged; however, the resulting derivative is more water-soluble.[16]

The first carbocyclic analogues of MDP, *N*-[D-2-(cyclohexyloxy)propionyl]-L-alanyl-D-isoglutamine (**3**) and two isomers of *N*-[2-[[2′-(acetylamino)-D-6′-hydroxycyclohexyl]oxy]propionyl]-L-alanyl-D-isoglutamine (**4**), were synthesized and tested by Hasegawa et al. to clarify the structural requirements in the carbohydrate moiety which are necessary for adjuvant activity.[29] These compounds in which the carbohydrate moiety of MDP was replaced with cyclohexanol derivatives were inactive as adjuvants for the induction of delayed-type hypersensitivity to azobenzenearsonate *N*-acetyl-L-tyrosine in guinea pigs.[30] This finding led the authors to the speculation that the carbohydrate moiety is essential for the adjuvant activity of MDP and its analogues.[30] Barton et al. reported the synthesis of

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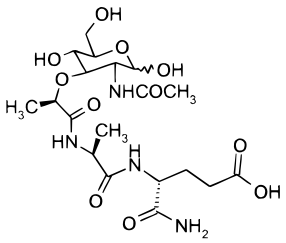
Chart 1



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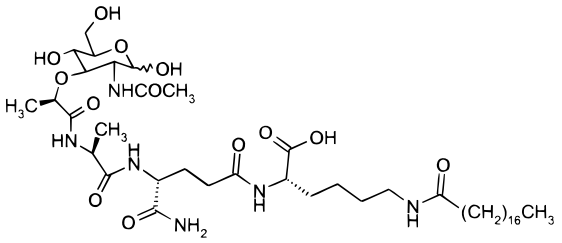

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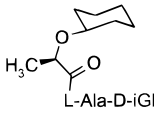

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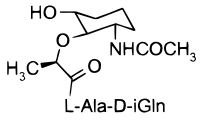

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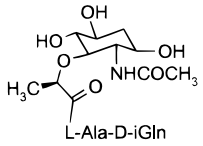
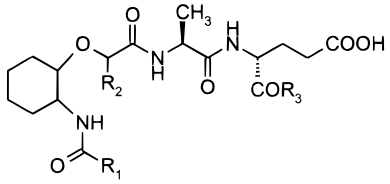


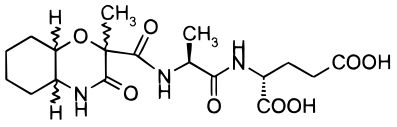
5

(1′*R*,2′*S*,3′*S*,5′*S*,6′*R*)-*N*-[D-2-[[2′-(acetylamino)-3′,5′,6′-trihydroxycyclohexyl]oxy]propionyl]-L-alanyl-D-isoglutamine (**5**) and some derivatives thereof[31] which exhibited the common activity of MDP, especially the stimulation of unspecific resistance against bacterial and viral infections, liberation of colony-stimulating factors, induction of IL-1 production in macrophages, and antitumor activity.[32]

To clarify the importance of the *N*-acetylmuramyl moiety of MDP for immunomodulatory activity, we prepared and studied in different immunological models novel carbocyclic MDP analogues of general structure **6**, obtained by replacement of the *N*-acetylmuramic acid part of the MDP molecule by a *trans*-2-[[2′-(acylamino)-cyclohexyl]oxy]acyl moiety, and **21**, resulting from further conformational restriction of the parent carbocyclic analogue *N*-{*trans*-2-[[2′-(acetylamino)cyclohexyl]oxy]-acetyl}-L-alanyl-D-glutamic acid (**9**) by incorporation of the lactoyl fragment and acetamido group into a cyclohexane-fused 1,4-morpholin-3-one ring. In the target compounds the trans orientation of the two substituents bound to a cyclohexane ring is similar to the orientation of the acetamido group and D-lactic acid moiety with respect to the glucopyranose ring in MDP. The replacement of a polyhydroxy-substituted pyranose ring of D-glucosamine in MDP by a more lipophilic cyclohexane ring should provide information about the importance of sugar hydroxyl groups for immunoadjuvant activity. The target molecules comprising several asymmetric centers in the acyl part were generally prepared as mixtures of diastereomers and, in some cases, to study the influence of chirality on their immunological activity, also in the form of pure diastereomers.

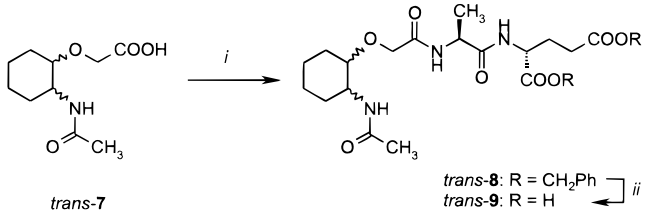


trans-**6**: R_1 = alkyl, R_2 = H,CH_3, R_3 = OH,NH_2



trans-**21**

Scheme 1[a]

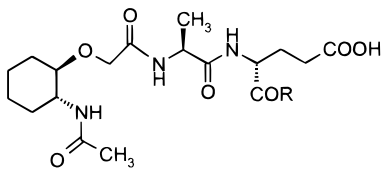


[a] (i) HCl·L-Ala-D-Glu$(OCH_2Ph)_2$, DPPA, Et_3N, DMF; (ii) H_2, Pd/C, MeOH.

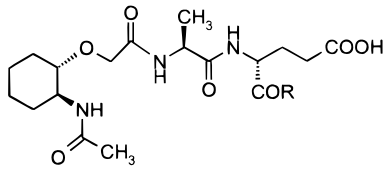
Results and Discussion

Chemistry. *N*-{*trans*-2-[[2′-(Acetylamino)cyclohexyl]-oxy]acetyl}-L-alanyl-D-glutamic acid (**9**) was obtained as a 1:1 mixture of 1′*R*,2′*R*- and 1′*S*,2′*S*-diastereomers by condensation of racemic *trans*-2-[[2′-(acetylamino)cyclohexyl]oxy]acetic acid (**7**)[33] with L-alanyl-D-glutamic acid dibenzyl ester[34] in dry *N*,*N*-dimethylformamide in the presence of diphenyl phosphorazidate (DPPA)[35−38] and triethylamine followed by hydrogenolytic cleavage of the benzyl-protecting groups in the resulting dipeptide **8** (Scheme 1). Pure diastereomers of **9**, (1′*R*,2′*R*)-*N*-{*trans*-2-[[2′-(acetylamino)cyclohexyl]oxy]acetyl}-L-alanyl-D-glutamic acid (**9a**) and (1′*S*,2′*S*)-*N*-{*trans*-2-[[2′-(acetylamino)cyclohexyl]oxy]acetyl}-L-alanyl-D-glutamic acid (**9b**) (Chart 2), as well as the respective isoglutamine analogues (1′*R*,2′*R*)-*N*-{*trans*-2-[[2′-(acetylamino)cyclohexyl]oxy]acetyl}-L-alanyl-D-isoglutamine (**10a**) and (1′*S*,2′*S*)-*N*-{*trans*-2-[[2′-(acetylamino)cyclohexyl]oxy]acetyl}-L-alanyl-D-isoglutamine (**10b**) (Chart 2) were prepared by analogy from enantiomers of carboxylic acid **7** or by chromatographic separation of the corresponding diastereomeric mixtures of benzyl-protected dipeptides as described by us previously.[39a] Four diastereomers of *N*-{*trans*-2-[[2′-(acetylamino)-cyclohexyl]oxy]propionyl}-L-alanyl-D-isoglutamine (**11a**−**d**) (Chart 2) were obtained similarly from respective

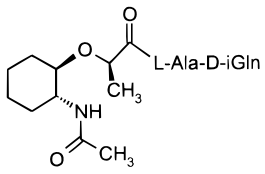
Chart 2



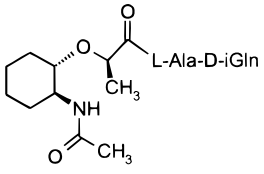
9a: R = OH
10a: R = NH_2



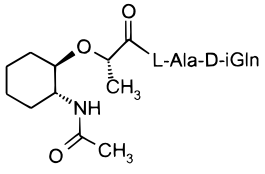
9b: R = OH
10b: R = NH_2



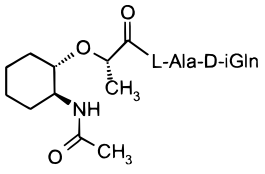
11a



11b

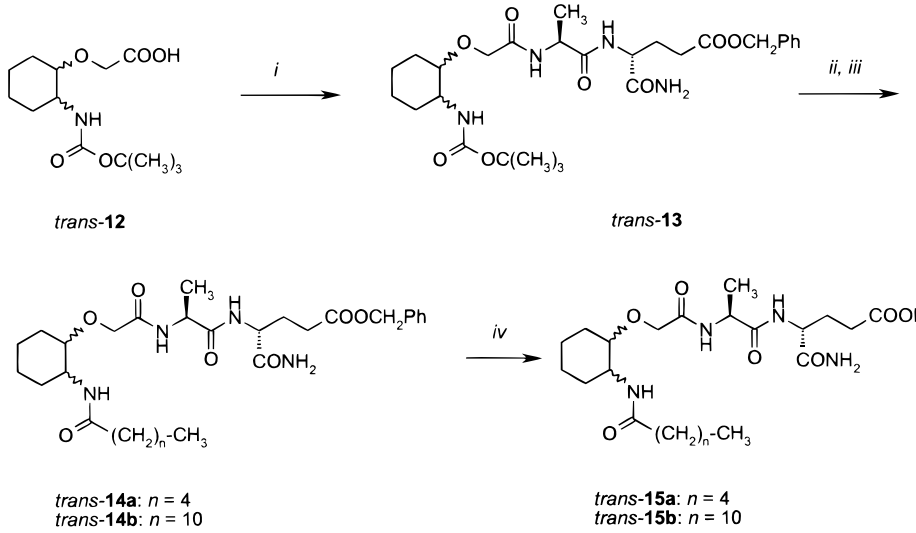


11c



11d

Scheme 2[a]



trans-**12**

trans-**13**

trans-**14a**: *n* = 4
trans-**14b**: *n* = 10

trans-**15a**: *n* = 4
trans-**15b**: *n* = 10

[a] (i) HCl·L-Ala-D-iGln(OCH_2Ph), DPPA, Et_3N, DMF; (ii) HCl (g), HOAc; (iii) $CH_3(CH_2)_nCOOH$, DPPA, Et_3N, DMF; (iv) H_2, Pd/C, MeOH.

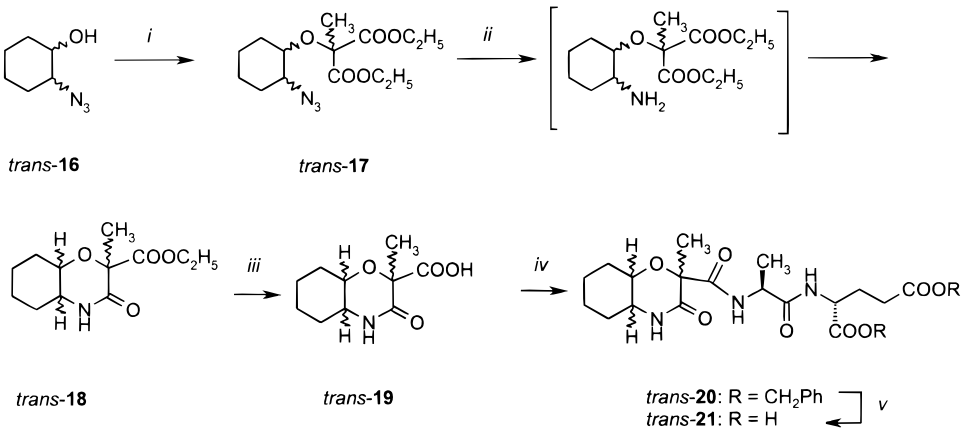
2*R*,1′*R*,2′*R*-, 2*S*,1′*R*,2′*R*-, 2*R*,1′*S*,2′*S*-, and 2*S*,1′*S*,2′*S*-isomers of 2-[[2′-(acetylamino)cyclohexyl]oxy]propionic acid.[39b]

For the preparation of *N*-{*trans*-2-[[2′-(hexanoylamino)cyclohexyl]oxy]acetyl}-L-alanyl-D-isoglutamine (**15a**) and *N*-{*trans*-2-[[2′-(dodecanoylamino)cyclohexyl]oxy]-acetyl}-L-alanyl-D-isoglutamine (**15b**), a different synthetic strategy was employed (Scheme 2). Thus, racemic *trans*-2-[[2′-(*tert*-butyloxycarbonylamino)cyclohexyl]oxy]-acetic acid (**12**)[33] was coupled with the benzyl ester of L-alanyl-D-isoglutamine[40,41] in the presence of DPPA to give benzyl *N*-{*trans*-2-[[2′-(*tert*-butyloxycarbonylamino)cyclohexyl]oxy]acetyl}-L-alanyl-D-isoglutaminate (**13**), which upon deprotection with hydrogen chloride in glacial acetic acid and condensation of the resulting benzyl *N*-[*trans*-2-[(2′-aminocyclohexyl)oxy]acetyl]-L-alanyl-D-isoglutaminate hydrochloride with hexanoic and dodecanoic acid, respectively afforded benzyl-protected dipeptides **14a**,**b**. Finally, hydrogenolysis of **14a**,**b** over 10% palladium on charcoal in methanol gave compounds **15a**,**b**, respectively, in almost quantitative yields.

The preparation of *N*-[(*trans*-2-methyl-3-oxooctahydro-2*H*-1,4-benzoxazin-2-yl)carbonyl]-L-alanyl-D-glutamic acid (**21**) is illustrated in Scheme 3. O-Alkylation of *trans*-2-azidocyclohexanol (**16**)[42] with diethyl 2-bromo-2-methylmalonate using sodium hydride in anhydrous dioxane as a base afforded *trans*-diethyl 2-[(2′-azidocyclohexyl)oxy]-2-methylmalonate (**17**) which was converted to *trans*-ethyl 2-methyl-3-oxooctahydro-2*H*-1,4-benzoxazine-2-carboxylate (**18**) on reduction of the azido group followed by spontaneous cyclization. Alkaline hydrolysis of **18** gave *trans*-2-methyl-3-oxooctahydro-2*H*-1,4-benzoxazine-2-carboxylic acid (**19**) which was coupled with dibenzyl L-alanyl-D-glutamate[34] in the presence of DPPA, and the resulting dipeptide derivative **20** was subsequently hydrogenolytically cleaved to give the target compound **21**.

Biological Data. 1. In Vivo Tests. Immunorestoration Test in Mice. The ability of novel carbocyclic MDP analogues to enhance nonspecific resistance to experimental fungal infection in immunosuppressed animals was examined in an immunorestoration test[43] in order to select a promising compound for further evaluation. It has already been reported that synthetic analogues of MDP increase nonspecific resistance to microbial infections mainly by exerting a priming effect on phagocytic cells leading to an enhancement in responses to the microbial challenge, such as microbi-

Scheme 3[a]



[a] (i) $Br(CH_3)C(COOC_2H_5)_2$, NaH, dioxane; (ii) $SnCl_2 \cdot 2H_2O$, MeOH; (iii) 1 N NaOH, dioxane; (iv) HCl·L-Ala-D-Glu$(OCH_2Ph)_2$, DPPA, Et_3N, DMF; (v) H_2, Pd/C, MeOH.

Table 1. Immunorestoration Test in Mice

		survival increase[a] [dose (mg/kg)]				
compd	configuration	3 × 0.1	3 × 1	3 × 10	other	formula[b]
9a	1′*R*,2′*R*	2/6	1/6	3/6	3/6[c]	$C_{18}H_{29}N_3O_8$
9b	1′*S*,2′*S*	0/6	1/6	2/6	2/6[c]	$C_{18}H_{29}N_3O_8$
10a	1′*R*,2′*R*	0/6	1/6	0/6	0/6[c]	$C_{18}H_{30}N_4O_7$
10b	1′*S*,2′*S*	0/6	0/6	0/6	1/6[c]	$C_{18}H_{30}N_4O_7 \cdot 1.5H_2O$
15a		0/6	0/6	1/6	1/6[d]	$C_{22}H_{38}N_4O_7$
15b		0/6	2/6	3/6	3/6[d]	$C_{28}H_{50}N_4O_7$[e]
21		0/6	0/6	0/6	2/6[c]	$C_{18}H_{27}N_3O_8$
MDP		0/6	3/6	2/6	4/6[c]	
azimexon					3/6[f]	

[a] $n_{actual}/n_{potential}$, where n_{actual} = (survival number for a group treated with test substance and cyclophosphamide) − (survival number for cyclophosphamide-treated group) and $n_{potential}$ = (survival number for blank control group) − (survival number for cyclophosphamide-treated group). [b] Satisfactory microanalyses for C,H,N obtained (±0.40%) except where indicated. [c] Dose: 3 × 100 mg/kg. [d] Dose: 3 × 30 mg/kg. [e] Calcd: C, 60.62; H, 9.09; N, 10.10. Found: C, 59.85; H, 9.17; N, 10.61. [f] The same value obtained in four independent experiments.

cidal activity and release of cytokines.[47] In the immunorestoration test the carbocyclic MDP analogues were tested in four different doses (0.1, 1.0, 10, and 30 or 100 mg/kg/day) in mice immunosuppressed with cyclophosphamide and infected with *Candida albicans*. Under these circumstances a 70−90% mortality was observed in the untreated immunosuppressed control group within 10 days. In the vehicle-treated control group in which the mice were not immunosuppressed with cyclophosphamide the mortality was 10−30% within 10 days. The immunorestorative activity was estimated as the survival increase in groups treated with the tested substances. Under the described experimental conditions, in a group of 10 mice treated with a test substance, the potential survival increase ($n_{potential}$) in all experiments was 6 animals (Table 1), and consequently, the standard statistical analysis (chi-square test) was unreliable. Therefore, the survival increase of 3 animals in any group treated with a test substance, which was also usually obtained by azimexon as the positive control compound, was considered significant and possibly a result of immunorestorant activity. The results of the immunorestoration test for compounds **9a**,**b**, **10a**,**b**, **15a**,**b**, and **21** as well as azimexon[43,50] and MDP as reference compounds are presented in Table 1. 2′-Acetylamino analogues **9a**,**b**, **10a**,**b**, and **11a**−**d** which were available in diastereomerically pure forms were tested as single diastereomers, whereas other compounds were assessed as equimolar mixtures of two diastereomers (**15a,b**) or four diastereomers (**21**), respectively. The 1′*R*,2′*R*-isomer of the D-glutamic acid derivative **9** displayed significant immunorestorant activity at doses of 10 and 100 mg/kg/day. Interestingly, both diastereomers of the corresponding D-isoglutamine derivative (**10a**,**b**) were markedly less active, although in the isoglutamine MDP analogues the adjuvant and antiinfectious activities are generally higher as compared to the glutamate series of analogues.[16] All four diastereomers of *N*-{*trans*-2-[[2′-(acetylamino)cyclohexyl]oxy]propionyl}-L-alanyl-D-isoglutamine (**11a-d**) were also inactive in the immunorestoration test at the dose of 10 mg/kg/day (results not shown), thus supporting a hypothesis that in this carbocyclic series of MDP analogues D-glutamic acid is a prerequisite for immunostimulating activity. Analogues with increased lipophilicity (**15a**,**b**) displayed immunostimulation at higher doses, and the immunostimulating activity was the most expressed in dodecanoylamino derivative **15b** where a dose-dependent response was achieved. The results obtained for a partially rigidified analogue (**21**) revealed that rigidification in the muramyl part of **9** is not beneficial for immunorestorative activity. In contrast to the solution conformation of MDP and some analogues,[51,52,53] compound **21** with fixed orientation of the acetamido carbonyl cannot form a β-turn by hydrogen bonding between the oxygen atom of the acetamido group and the amino group of the L-alanyl moiety. It has been proposed that any structural change performed in MDP modifying the β-turn can produce measurable

Table 2. Effect of Compound **9** on the Number of Total Spleen Cells, B Cells, T Cells, and Peritoneal Macrophages (in Millions)

		total spleen cells		B cells		T cells		peritoneal macrophages	
compd	daily dose/ mouse (μg)	$X \pm$ SEM	p	$X \pm$ SEM	p	$X \pm$ SEM	p	$X \pm$ SEM	p
MDP	25	12.4 ± 6.32	0.549	24.18 ± 4.69	0.979	13.64 ± 4.49	0.913	2.11 ± 0.64	0.47
	2.5	9 ± 5.66	0.348	20.94 ± 2.71	0.358	11.92 ± 1.67	0.636	3.67 ± 2.34	0.076
	0.25	10.6 ± 6.13	0.843	19.34 ± 6.05	0.231	13.32 ± 4.59	0.709	1.88 ± 1.09	0.923
9	25	9.93 ± 6.02	0.694	20.5 ± 11.06	0.48	9.72 ± 2.57	0.295	0.92 ± 0.61	0.114
	2.5	8.14 ± 3.38	0.361	23.76 ± 10.21	0.945	12.88 ± 6.47	0.848	2.87 ± 2.03	0.418
	0.25	5.66 ± 3.31	0.07	18.42 ± 9.57	0.234	11.7 ± 9.98	0.401	2.58 ± 2.23	0.976
control		11.42 ± 4.99		24.08 ± 7		14.05 ± 8.32		1.6 ± 2.2	

Table 3. Immunomodulatory Effect of MDP and Compound **9** on Blast Transformation Ability of Lymphocytes, Stimulated in Vitro with Concanavalin A

		amount of [^{3}H]thymidine incorporated in lymphocyte DNA[a]	
compd	daily dose (μg)[b]	X ± SEM (n)	p
MDP	25	7460 ± 1980 (5)	>0.05
	2.5	12440 ± 3540 (5)	>0.05
	0.25	19840 ± 9400 (5)	>0.05
9	25	25990 ± 7850 (5)	<0.05
	2.5	16830 ± 6610 (5)	>0.05
	0.25	10780 ± 2680 (5)	>0.05
control		10130 ± 476 (5)	

[a] cpm. [b] Said doses were administered to animals for 3 consecutive days.

variation of the biological activity.[53] Therefore, reduced activity of the rigidified analogue **21** could be explained by its different conformation in aqueous solution. From the results obtained it can be concluded that compounds **9a**,**b** and **15b** protect mice against the immunosuppressive effect of cyclophosphamide. Because cyclophosphamide also impairs the function of lymphocytes[44] and accessory immune system cells[45,46] the beneficial effect of compounds **9** and **15** can result from their effect on these cells. A possible direct antifungal activity of the tested substances was excluded by in vitro testing of compound **15b** which was one of the most active substances in the immunorestoration test against *C. albicans*. The negative result of this test ruled out a possible direct antimicrobial activity of this compound. On the basis of results of the in vivo testing and considering the synthetic availability of various tested carbocyclic MDP analogues, we decided to investigate in detail in several immunopharmacological models the analogue **9** as a diastereomeric mixture since no significant difference in the immunorestorative activity of 1′*R*,2′*R*- and 1′*S*,2′*S*-diastereomers could be established.

2. In Vivo/in Vitro Tests. Neither the number of splenic lymphocytes B and lymphocytes T nor the number of peritoneal macrophages was statistically significantly altered by either MDP or compound **9** (Table 2).

A modified hemolytic plaque-forming cell assay[48] for the determination of maturation of lymphocytes B revealed that B lymphocytes may be important target cells for compound **9**. A significant increase ($p < 0.001$) of the number of plaques from 136.2 ± 17.0 ($n = 12$) to 331.6 ± 25.3 ($n = 13$) was observed at the dose of 1 μg of **9**/mouse. The stimulation of B cell maturation to plasma cells by **9** was comparable to the effect of MDP.[27a]

Studies of concanavalin A (Con A)-induced blast transformation of lymphocytes led to the conclusion that MDP did not affect the blastogenic activity of lymphocytes T, whereas compound **9** at the dose of 25 μg increased this function by almost 150% ($p < 0.05$). This effect was dose-dependent. In comparison with MDP, compound **9** gave in this test a statistically significantly greater ($p < 0.05$) immunomodulatory effect in lymphocytes T at the dose of 25 μg (Table 3).

In studies of macrophage activation, the production of O_2^- demonstrated an increased effectiveness of all three doses of MDP by about 120% ($p < 0.001$), whereas compound **9** increased this activity at the dose of 2.5 μg by 55% ($p < 0.06$) and at the dose of 25 μg by 70% ($p < 0.005$). For compound **9** the effects were dose-dependent (Table 4). Similar results were found after additional macrophage activation with phorbol myristate acetate (PMA). In this case muramyl dipeptide at all doses increased the activity by about 150% ($p < 0.001$), whereas compound **9** increased the activity at the dose of 2.5 μg by 50% ($p < 0.05$) and at the dose of 25 μg by 70%. Also in this case the effects were dose-dependent.

Determination of pyrogenicity of compound **9** according to the method of USP XXII showed that in contrast to MDP, compound **9** does not display any pyrogenic activity. This result may suggest that in comparison to MDP, the absence of pyrogenicity in **9** is probably a result of a missing carbohydrate moiety. The average lethal dose (LD_{50}) for iv application of compound **9** in male mice was found to be higher than 500 mg/kg.

Table 4. Immunomodulatory Effect of MDP and Compound **9** Upon Macrophage Activation Without or With Costimulation with PMA

		difference in absorbancies of the test sample and blank per mg of cell protein			
		without costimulation with PMA		with costimulation with PMA	
substance	daily dose (μg)[a]	$X \pm$ SEM (n)	p	$X \pm$ SEM (n)	p
MDP	25	578 ± 81 (5)	<0.01	2138 ± 165 (5)	<0.001
	2.5	578 ± 42 (5)	<0.001	2207 ± 213 (5)	<0.001
	0.25	614 ± 35 (5)	<0.001	2657 ± 595 (5)	<0.05
9	25	489 ± 97 (5)	<0.05	1709 ± 374 (5)	<0.05
	2.5	431 ± 85 (5)	<0.06	1494 ± 199 (5)	<0.05
	0.25	237 ± 94 (5)	>0.05	1298 ± 343 (5)	>0.05
control		275 ± 26 (5)		1004 ± 38 (5)	

[a] Said doses were administered to animals for 3 consecutive days.

In conclusion, a carbocyclic *nor*-MDP analogue (**9**) in which the *N*-acetylmuramyl moiety is replaced by a *trans*-2-[[2′-(acetylamino)cyclohexyl]oxy]acetyl group and D-isoglutamine is exchanged for D-glutamic acid retains the immunostimulating properties of MDP.[39c] On the basis of the performed immunobiological tests, we conclude that compound **9** protects mice against the immunosuppressive effect of cyclophosphamide and increases the nonspecific resistance of mice against fungal infection. Compound **9** is an immunomodulator which enhances the maturation of lymphocytes B to plasma cells and increases the activity of lymphocytes B and lymphocytes T as well as that of macrophages but does not alter the number of these cells. In the tested range the effects are dose-dependent. It has been already reported that as an immunomodulator compound **9** also displays antitumor activity; when combined with the tumor necrosis factor, it increases the activity and decreases the side effects of the latter.[49] Similar effects were also demonstrated for muramyl dipeptide,[3−8] but in contrast to MDP compound **9** is neither pyrogenic nor toxic.

Experimental Section

Chemistry. All reagents and solvents were of commercial grade and used as such unless specified. MDP was purchased from Bachem, Switzerland. Melting points were determined using a Reichert hot-stage microscope and are uncorrected. IR spectra were recorded on a Perkin-Elmer FTIR 1600 instrument. NMR spectra were recorded on a Varian VXR-300 spectrometer with tetramethylsilane as internal standard. Mass spectra were recorded on a VG-Analytical Autospec Q mass spectrometer. Microanalyses were carried out at the Department of Organic Chemistry, Faculty of Chemistry and Chemical Technology, Ljubljana, on a Perkin-Elmer elemental analyzer 240 C. Optical rotations were determined on a Perkin-Elmer polarimeter 1241 MC using a 1-dm cell. HPLC analysis was performed on a Hewlet-Packard 1090 system with a Licrospher 100 RP18 5-μm (150 × 4.6-mm) column. The mobile phase was a linear gradient from 0.1% trifluoroacetic acid in water to 60% acetonitrile and 0.1% trifluoroacetic acid in water. For preparative purification a semipreparative column, Microsorb C18 3 μm (50 × 22 mm), was used.

Dibenzyl *N*-{*trans*-2-[[2′-(Acetylamino)cyclohexyl]-oxy]acetyl}-L-alanyl-D-glutamate (8). Standard Procedure A: To a stirred solution of dibenzyl L-alanyl-D-glutamate hydrochloride (869 mg, 2 mmol) and the acid **7** (430 mg, 2 mmol) in dry DMF (10 mL) were added at 0−2 °C DPPA (550 mg, 2 mmol) and Et_3N (0.56 mL, 4 mmol). The mixture was stirred for 1 h at 0−2 °C and then for 60 h at room temperature. Ethyl acetate (40 mL) was added, and the mixture was extracted successively with 10% citric acid (3 × 5 mL), H_2O (3 × 5 mL), saturated NaCl solution (3 × 5 mL), saturated $NaHCO_3$ solution (3 × 5 mL), H_2O (3 × 5 mL), and saturated NaCl solution (3 × 5 mL). The combined organic extracts were dried over $MgSO_4$, filtered, and evaporated in vacuo. The crude product was purified by column chromatography on silica gel using $CHCl_3$/MeOH (9/1) as eluent to give **8** as a colorless viscous oil: yield 1.0 g (84%); IR (film) 3285, 3066, 2934, 2858, 1744, 1649, 1548, 1446, 1374, 1271, 1169, 966, 753, 696, 578 cm^{-1}; UV (CH_3OH) λ_{max} (log ϵ) = 202 nm (4.42); ^{1}H NMR ($CDCl_3$) δ 1.00−1.38 (m, 4H, $4H_{ax}$), 1.38 (1.39) (d, 3H, J = 7.0 Hz, CH_3-Ala), 1.60−1.80 (m, 2H, $2H_{eq}$), 1.90−2.15 (m, 4H, $2H_{eq}$, CH_2-βGlu), 1.95 (1.96) (s, 3H, $COCH_3$), 2.41 (t, 2H, CH_2-γGlu), 3.0−3.10 (m, 1H, 1′-H), 3.70−3.90 (m, 1H, 2′-H), 3.97 (3.99) (AB system, 2H, J = 15.1 Hz, OCH_2), 4.40−4.63 (m, 2H, CH-Glu, CH-Ala), 5.09 (s, 2H, CH_2-benzyl), 5.14 (s, 2H, CH_2-benzyl), 6.15 (6.62) (d, 1H, J = 8.0 Hz, NH), 7.20−7.44 (m, 12H, $10H_{arom}$, 2NH); MS m/z 595 (M^+, 6), 241 (100); Anal. ($C_{32}H_{41}N_3O_8$) C, H, N.

***N*-{*trans*-2-[[2′-(Acetylamino)cyclohexyl]oxy]acetyl}-L-alanyl-D-glutamic Acid (9).** Dibenzyl ester **8** (1.18 g, 1.98 mmol) was dissolved in MeOH (20 mL) and hydrogenated over 10% Pd/C (160 mg) for 1 h at room temperature and normal pressure. The catalyst was removed by filtration, and the filtrate was evaporated in vacuo. There was obtained 820 mg (99%) of **9** in the form of a white amorphous solid foam: IR (KBr) 3340, 3085, 2940, 2900−2300 br, 1734, 1654, 1542, 1449, 1375, 1213, 1115, 970, 856, 670, 590 cm^{-1}; UV (CH_3OH) λ_{max} (log ϵ) = 202 nm (3.79); ^{1}H NMR (DMSO-d_6) δ 1.0−1.24 (m, 4H, $4H_{ax}$), 1.19 (d, 3H, J = 6.9 Hz, CH_3-Ala), 1.55−1.70 (m, 2H, $2H_{eq}$), 1.70−1.90 (m, 2H, $1H_{eq}$, H-βGlu), 1.80 (1.83) (s, 3H, $COCH_3$), 1.95−2.15 (m, 2H, $1H_{eq}$, H-βGlu), 2.24 (t, 2H, J = 7.1 Hz, CH_2-γGlu), 3.10−3.20 (m, 1H, 1′-H), 3.50−3.70 (m, 1H, 2′-H), 3.87 (3.93) (AB system, 2H, J = 15.3 Hz, OCH_2), 4.18−4.30 (m, 1H, CH-Glu), 4.35−4.42 (m, 1H, CH-Ala), 7.54 (7.56) (d, 1H, J = 7.8 Hz, NH), 7.94 (d, 1H, J = 7.8 Hz, NH), 8.33 (8.35) (d, 1H, J = 7.9 Hz, NH), 12.4 (s br, 2H, 2 COOH); MS m/z 415 (M^+, <0.01), 84 (100). Anal. ($C_{18}H_{29}N_3O_8\times0.5H_2O$) C, H, N.

Benzyl *N*-{*trans*-2-[[2′-(*tert*-Butyloxycarbonylamino)-cyclohexyl]oxy]acetyl}-L-alanyl-D-isoglutaminate (13). Using standard procedure A, condensation of racemic carboxylic acid **12**[33] (0.900 g, 3.29 mmol) and the hydrochloride of L-alanyl-D-isoglutamine benzyl ester[40,41] (1.131 g, 3.29 mmol) afforded **13** as a solid white amorphous foam: yield 1.60 g (86%); IR (KBr) 3700−3100 br, 3066, 2937, 2861, 1730, 1684, 1534, 1452, 1366, 1321, 1256, 1169, 1114, 1014, 698 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.00−1.50 (m, 4H, $4H_{ax}$), 1.23 (1.24) (d, 3H, J = 6.8 Hz, CH_3-Ala), 1.36 (s, 9H, $C(CH_3)_3$), 1.50−1.68 (m, 2H, $2H_{eq}$), 1.70−1.90 (m, 2H, CH_2-βiGln), 1.90−2.10 (m, 2H, $2H_{eq}$), 2.36 (t, 2H, J = 7.6 Hz, CH_2-γiGln), 3.30−3.32 (m, 2H, 1′-H, 2′-H), 3.93 (AB system, 2H, J = 15.6 Hz, OCH_2), 4.15−4.28 (m, 1H, CH-iGln), 4.28−4.40 (m, 1H, CH-Ala), 5.08 (s, 2H, CH_2-benzyl), 6.85 (6.93) (d, 1H, J = 7.5 Hz, NH), 7.13 (s, 1H, NH), 7.22−7.50 (m, 6H, $5H_{arom}$, NH), 7.55 (7.61) (d, 1H, J = 7.0 Hz, NH), 8.23 (8.27) (d, 1H, J = 8.2 Hz, NH). Anal. ($C_{28}H_{42}N_4O_8$) C, H, N.

Benzyl *N*-{*trans*-2-[[2′-(Hexanoylamino)cyclohexyl]-oxy]acetyl}-L-alanyl-D-isoglutaminate (14a). A slow stream of dry hydrogen chloride gas was passed for 30 min through a solution of **13** (1.51 g, 2.68 mmol) in glacial acetic acid (10 mL). HOAc was removed in vacuo and the residue was triturated with dry diethyl ether to give benzyl *N*-[*trans*-2-[(2′-aminocyclohexyl)oxy]acetyl]-L-alanyl-D-isoglutaminate hydrochloride as a white amorphous hygroscopic solid which was used in the next step without further purification: yield 1.20 g (90%); IR (KBr) 3700−3100 br, 3052, 2928, 2857, 1730, 1655, 1540, 1451 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.00−1.50 (m, 4H, $4H_{ax}$), 1.28 (d, 3H, J = 6.89 Hz, CH_3-Ala), 1.50−1.70 (m, 2H, $2H_{eq}$), 1.70−1.90 (m, 1H, CH_2-βiGln), 1.95−2.20 (m, 3H, $2H_{eq}$, CH_2-βiGln), 2.37 (t, 2H, J = 7.76 Hz, CH_2-γiGln), 2.85−3.00 (m, 1H, 1′-H/ 2′-H), 3.30−3.45 (m, 1H, 1′-H/ 2′-H), 4.00 (4.01) [AB system, 2H, $J_{A,B}$ = 15.1 Hz (J_{AB} = 15.2 Hz), OCH_2], 4.15−4.26 (m, 1H, CH-iGln), 4.25−4.42 (m, 1H, CH-Ala), 5.09 (s, 2H, CH_2-benzyl), 7.12 (s br, 1H, $CONH_2$), 7.30−7.45 (m, 6H, $5H_{arom}$, $CONH_2$), 8.21 (8.38) (d, 1H, J = 7.13 Hz, NH), 8.28 (d, 1H, J = 7.62 Hz, NH), 8.37 (s br, 3H, NH_3^+). Anal. ($C_{23}H_{35}ClN_4O_6$) Calcd: C, 55.36; H, 7.07; N, 11.23. Found: C, 54.96; H, 7.10; N, 10.88.

Using standard procedure A, hexanoic acid (139 mg, 1.2 mmol) and benzyl *N*-[*trans*-2-[(2′-aminocyclohexyl)oxy]acetyl]-L-alanyl-D-isoglutaminate hydrochloride (599 mg, 1.2 mmol) were condensed to give **14a** as a white solid amorphous foam: yield 504 mg (75%); IR (KBr) 3700−3350 br, 3284, 2919, 2861, 1731, 1654, 1631, 1543, 1449, 1167, 1096 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.78 (0.79) (t, 3H, J = 6.64 Hz, CH_3), 1.18 (d, 3H, J = 7.03 Hz, CH_3-Ala), 0.95−1.35 (m, 8H, $4CH_2$), 1.35−1.85 (m, 6H, $3CH_2$), 1.85−2.10 (m, 4H, $2CH_2$), 2.32 (t, 2H, J = 7.69 Hz, CH_2-γiGln), 3.02−3.16 (m, 1H, 1′-H/ 2′-H), 3.45−3.60 (m, 1H, 1′-H/ 2′-H), 3.86 (3.87) [AB system, 2H, $J_{A,B}$ = 15.17 Hz (J_{AB} = 15.16 Hz), OCH_2], 4.05−4.20 (m, 1H, CH-iGln), 4.20−4.32 (m, 1H, CH-Ala), 5.03 (s, 2H, CH_2-benzyl), 7.08 (s br, 1H, $CONH_2$), 7.21−7.42 (m, 6H, $5H_{arom}$, $CONH_2$), 7.52 (7.56) [d, 1H, J = 6.78 Hz (J = 7.08 Hz), NH], 7.77 (7.78) [d, 1H, J

= 7.68 Hz (J = 7.62 Hz), NH], 8.17 (8.21) [d, 1H, J = 8.08 Hz (J = 8.11 Hz), NH]. Anal. ($C_{29}H_{44}N_4O_7$) C, H, N.

Benzyl *N*-{*trans*-2-[[2′-(Dodecanoylamino)cyclohexyl]oxy]acetyl}-L-alanyl-D-isoglutaminate (14b). Using standard procedure A, dodecanoic acid (220 mg, 1.1 mmol) and benzyl *N*-[*trans*-2-[(2′-aminocyclohexyl)oxy]acetyl]-L-alanyl-D-isoglutaminate hydrochloride (549 mg, 1.1 mmol) were condensed to give **14b** as a white solid amorphous foam: yield 496 mg (70%); IR (KBr) 3700−3350 br, 3285, 2924, 2853, 1735, 1654, 1543, 1450, 1268, 1169 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.83 (t, 3H, J = 6.89 Hz, CH_3), 1.00−1.35 (m, 23H, 10CH_2, CH_3-Ala), 1.35−1.50 (m, 2H, CH_2), 1.55−1.66 (m, 2H, CH_2), 1.68−1.85 (m, 2H, CH_2), 1.90−2.15 (m, 4H, 2CH_2), 2.35 (t, 2H, J = 7.74 Hz, CH_2-γiGln), 3.05−3.18 (m, 1H, 1′-H/ 2′-H), 3.45−3.62 (m, 1H, 1′−H/ 2′-H), 3.89 (3.90) [AB system, 2H, $J_{A,B}$ = 15.23 Hz ($J_{A,B}$ = 15.25 Hz), OCH_2], 4.12−4.22 (m, 1H, CH-iGln), 4.24−4.36 (m, 1H, CH-Ala), 5.07 (s, 2H, CH_2-benzyl), 7.12 (s br, 1H, $CONH_2$), 7.27−7.41 (m, 6H, 5H_{arom}, $CONH_2$), 7.55 (7.59) [d, 1H, J = 7.08 Hz (J = 7.03 Hz), NH], 7.79 (7.80) [d, 1H, J = 7.87 Hz (J = 7.81 Hz), NH], 8.19 (8.25) [d, 1H, J = 8.06 Hz (J = 8.25 Hz), NH]. Anal. ($C_{35}H_{56}N_4O_7$) C, H, N.

***N*-{*trans*-2-[[2′-(Hexanoylamino)cyclohexyl]oxy]acetyl}-L-alanyl-D-isoglutamine (15a).** Benzyl ester **14a** (356 mg, 0.64 mmol) was hydrogenated in tetrahydrofuran (20 mL) over 10% Pd/C (54 mg) for 1 h at room temperature. The catalyst was removed by filtration, and the filtrate was evaporated in vacuo to give 258 mg (85%) of **15a** as a white amorphous solid foam: mp 177−181 °C; IR (KBr) 3436, 3295, 2931, 2861, 1725, 1649, 1543, 1449, 1120 cm^{-1}; UV (CH_3OH) λ_{max} (log ϵ) = 203 nm (3.89), 270 nm (3.55); ^{1}H NMR (DMSO-d_6) δ 0.84 (t, 3H, J = 6.93 Hz, CH_3), 1.04−1.32 (m, 9H, 3CH_2, CH_3−Ala), 1.40−1.86 (m, 8H, 4CH_2), 1.88−2.12 (m, 4H, 2CH_2), 2.21 (t, 2H, J = 7.69 Hz, CH_2-γiGln), 3.08−3.20 (m, 1H, 1′-H/ 2′-H), 3.50−3.64 (m, 1H, 1′-H/ 2′-H), 3.91 (3.92) [AB system, 2H, $J_{A,B}$ = 15.14 Hz ($J_{A,B}$ = 15.4 Hz), OCH_2], 4.12−4.24 (m, 1H, CH-iGln), 4.26−4.40 (m, 1H, CH-Ala), 7.11 (s br, 1H, $CONH_2$), 7.34 (s br, 1H, $CONH_2$), 7.56 (7.60) [d, 1H, J = 7.03 Hz (J = 7.08 Hz), NH], 7.81 (7.82) [d, 1H, J = 7.57 Hz (J = 7.57 Hz), NH], 8.20 (8.22) [d, 1H, J = 8.30 Hz (J = 8.11 Hz), NH], 12.16 (s br, 1H, COOH). Anal. ($C_{22}H_{38}N_4O_7$) C, H, N.

***N*-{*trans*-2-[[2′-(Dodecanoylamino)cyclohexyl]oxy]acetyl}-L-alanyl-D-isoglutamine (15b).** Benzyl ester **14b** (216 mg, 0.335 mmol) was hydrogenated in tetrahydrofuran (15 mL) over 10% Pd/C (32 mg) for 1 h at room temperature. The catalyst was removed by filtration, and the filtrate was evaporated in vacuo to give 179 mg (96%) of **15b** as a white amorphous solid: mp 171−175 °C; IR (KBr) 3438, 3292, 2926, 2853, 1730, 1648, 1552, 1448, 1274, 1196, 1123 cm^{-1}; UV (CH_3-OH) λ_{max} (log ϵ) = 203 nm (3.91), 270 nm (3.38); ^{1}H NMR (DMSO-d_6) δ 0.85 (t, 3H, J = 6.62 Hz, CH_3), 1.00−1.35 (m, 23H, 10CH_2, CH_3-Ala), 1.35−1.85 (m, 6H, 3CH_2), 1.90−2.12 (m, 4H, 2CH_2), 2.20 (t, 2H, J = 7.76 Hz, CH_2-γiGln), 3.12−3.20 (m, 1H, 1′-H/2′-H), 3.45−3.65 (m, 1H, 1′-H/2′-H), 3.90 (3.92) [AB system, 2H, $J_{A,B}$ = 15.16 Hz (J_{AB} = 15.35 Hz), OCH_2], 4.10−4.22 (m, 1H, CH-iGln), 4.24−4.38 (m, 1H, CH-Ala), 7.11 (s br, 1H, $CONH_2$), 7.34 (s br, 1H, $CONH_2$), 7.56 (7.60) [d, 1H, J = 7.08 Hz (J = 6.89 Hz), NH], 7.80 (7.82) [d, 1H, J = 7.43 Hz (J = 7.57 Hz), NH], 8.19 (8.24) [d, 1H, J = 8.30 Hz (J = 8.30 Hz), NH], 12.10 (s br, 1H, COOH). Anal. ($C_{28}H_{50}N_4O_7$) Calcd: C, 60.62; H, 9.09; N, 10.10. Found: C, 59.85; H, 9.17; N, 10.61.

***trans*-Diethyl 2-[(2′-Azidocyclohexyl)oxy]-2-methylmalonate (17).** *trans*-2-Azidocyclohexanol (10.6 g, 75 mmol) was added dropwise at 70 °C to a magnetically stirred suspension of NaH (1.8 g, 75 mmol) in anhydrous dioxane (75 mL) in a three-necked flask equipped with a water-cooled reflux condenser. The reaction mixture was stirred at 70 °C for 1.5 h whereupon diethyl 2-bromo-2-methylmalonate (19 g, 75 mmol) was added dropwise, and the resulting mixture was stirred for an additional 4 h at 70−80 °C. After evaporation of the solvent, water (200 mL) was added and the resulting mixture was extracted with ethyl acetate (5 × 50 mL). The combined organic phases were dried over $MgSO_4$, filtered, and evaporated in vacuo. The crude product was distilled, and the fraction which distilled at 120−150 °C/0.20−0.67 mbar was further purified by column chromatography (silica gel, $CHCl_3$/MeOH = 100/1) to give 6.72 g (28%) of **17** as a pale-yellow viscous liquid: IR (film) 2980, 2940, 2860, 2110, 1740, 1450, 1375, 1270, 1150, 1115, 1060, 1025, 860 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.289 (1.287) (t, 6H, J = 7.1 Hz, 2COOCH_2CH_3), 1.20−1.50 (m, 4H, 4H_{ax}), 1.68 (s, 3H, 2-CH_3), 1.62−2.20 (m, 4H, 4H_{eq}), 3.36−3.48 (m, 1H, 1′-H/ 2′-H), 3.54−3.68 (m, 1H, 1′-H/ 2′-H), 4.25 (4.24) (q, 4H, J = 7.1 Hz, 2COOCH_2CH$_3$). Anal. ($C_{14}H_{23}N_3O_5$) C, H, N.

***trans*-Ethyl 2-Methyl-3-oxooctahydro-2*H*-1,4-benzoxazine-2-carboxylate (18)**. To a stirred solution of $SnCl_2$·$2H_2O$ (4.06 g, 18 mmol) in methanol (12 mL) was added a solution of **17** (3.76 g, 12 mmol) in methanol (12 mL) dropwise at 0−5 °C. The mixture was stirred overnight at room temperature, the solvent was evaporated, and the residue was stirred for 0.25 h with saturated Na_2CO_3 solution (75 mL). The mixture was extracted with diethyl ether (5 × 50 mL), and the combined organic extracts were dried over $MgSO_4$, filtered, and evaporated in vacuo. The residue was recrystallized from ethyl acetate to give **18** in the form of white crystals: yield 1.88 g (65%); mp 92−94 °C; IR (KBr) 3190, 3080, 2980, 2940, 2880, 1740, 1670, 1465, 1450, 1420, 1385, 1360, 1340, 1240, 1130, 1080, 1020, 965, 865, 810, 780, 730 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.29 (t, 3H, J = 7.1 Hz, COOCH_2CH_3), 1.20−2.00 (m, 8H, 4CH_2), 1.68 (s, 3H, 2-CH_3), 3.22−3.42 (m, 2H, 4a-H, 8a-H), 4.23 (m, 2H, J = 7.1 Hz, COOCH_2CH$_3$), 7.15 (s br, 1H, NH); ^{13}C NMR (75.44 MHz, $CDCl_3$) δ 13.87, 20.21, 23.23, 24.13, 29.74, 30.33, 55.76, 61.91, 75.07, 81.46, 169.11, 169.34; MS m/z (EI) 241 (M^+). Anal. ($C_{12}H_{19}NO_4$) C, H, N.

***trans*-2-Methyl-3-oxooctahydro-2*H*-1,4-benzoxazine-2-carboxylic Acid (19).** A solution of **18** (4.80 g, 19.9 mmol) and 1 N NaOH (20.7 mL, 20.7 mmol) in dioxane (80 mL) was stirred for 20 h at room temperature. The solvent was removed in vacuo, 1 N HCl (90 mL, 90 mmol) was added to the residue, and the mixture was extracted with ethyl acetate (4 × 60 mL). The organic phase was dried over $MgSO_4$, the solvent was evaporated, and the crude product was recrystallized from ethyl acetate: yield 2.3 g (54%), white crystals; mp 142−144 °C; IR (KBr) 3280, 2940, 2870, 2500, 1930, 1730, 1630, 1460, 1450, 1420, 1380, 1350, 1315, 1300, 1275, 1180, 1165, 1150, 1115, 1080, 975, 930, 880, 850, 810, 760, 710 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.20−2.15 (m, 8H, 4CH_2), 1.70 (1.68) (s, 3H, 2-CH_3), 3.10−3.15 (3.15−3.20) (m, 1H, 4a-H/8a-H), 3.50−3.62 (3.40−3.50) (m, 1H, 4a-H/8a-H), 7.65 (7.70) (s br, 1H, NH), 11.2 (s br, 1H, COOH); ^{13}C NMR (75.44 MHz, $CDCl_3$) δ 21.56, 23.36, 24.00, 29.60, 30.22, 56.40, 75.87, 80.11, 171.26, 171.75; MS m/z (EI) 213 (M^+). Anal. ($C_{10}H_{15}NO_4$) C, H, N.

***trans*-Dibenzyl *N*-[(2-Methyl-3-oxooctahydro-2*H*-1,4-benzoxazin-2-yl)carbonyl]-L-alanyl-D-glutamate (20). 20** was prepared from **19** (319 mg, 1.5 mmol) and dibenzyl L-alanyl-D-glutamate hydrochloride[34] (652 mg, 1.5 mmol) using standard procedure A; yield 770 mg (87%) of crude **20**. The crude product was purified by column chromatography (silica gel, $CHCl_3$/MeOH = 9:1) to give 720 mg (81%) of pure **20** as a white amorphous foam: IR (film) 3289, 2937, 2864, 1737, 1676, 1508, 1454, 1376, 1168, 739, 698, 533 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.00−1.38 (m, 4H, 4H_{ax}), 1.22 (1.23) (d, 3H, J = 6.8 Hz, CH_3-Ala), 1.46 (1.47) (s, 3H, 2-CH_3), 1.50−1.75 (m, 4H, 4H_{eq}), 1.75−1.98 (m, 1H, CH_2-βGlu), 1.98−2.16 (m, 1H, CH_2-βGlu), 2.36−2.48 (m, 2H, CH_2-γGlu), 3.00−3.25 (m, 1H, 4a-H/8a-H), 3.30−3.50 (m, 1H, 4a-H/8a-H), 4.26−4.44 (m, 2H, CH-Glu, CH-Ala), 5.08 (s, 2H, CH_2-benzyl), 5.13 (s, 2H, CH_2-benzyl), 7.28−7.44 (m, 10H, H_{arom}), 7.65−8.50 (m, 3H, 3NH); MS m/z (FAB) 594 (MH^+). Anal. ($C_{32}H_{39}N_3O_8$) C, H, N.

***trans*-*N*-[(2-Methyl-3-oxooctahydro-2*H*-1,4-benzoxazin-2-yl)carbonyl]-L-alanyl-D-glutamic Acid (21).** Hydrogenation of dibenzyl ester **20** (700 mg, 1.18 mmol) over 10% Pd/C (110 mg) in methanol (20 mL) for 1 h at normal pressure and room temperature afforded **21** as a white amorphous solid: yield 453 mg (93%); IR (KBr) 3318, 2843, 1737, 1668, 1521, 1450, 1375, 1216, 1169, 1110, 1069, 957, 634 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.00−1.40 (m, 7H, 4H_{ax}, CH_3-Ala), 1.44

(1.46) (s, 3H, 2-CH_3), 1.50−2.10 (m, 6H, 4H_{eq}, CH_2-βGlu), 2.10−2.22 (m, 2H, CH_2-γGlu), 3.00−3.24 (m, 1H, 4a-H/8a-H), 3.24−3.55 (m, 1H, 4a-H/8a-H), 4.26−4.40 (m, 2H, CH-Glu, CH-Ala), 7.62−8.50 (m, 3H, 3NH), 12.50 (s br, 2H, 2COOH). Anal. ($C_{18}H_{27}N_3O_8$) Calcd: C, 52.29; H, 6.58; N, 10.16. Found: C, 51.83; H, 6.87; N, 9.99.

Pharmacology. 1. In Vivo Tests. Immunorestoration Test. For the immunorestoration test, ICR-derived female mice, aged 6 weeks, 23−27 g in weight, were used. Animals were housed in polycarbonate cages containing wood shavings and covered with a stainless steel wire mesh. Groups of 10 mice were housed in cages measuring 45 cm in length by 23 cm in width and 15 cm in height. Animals were maintained in a positive pressure room with air filtration, controlled temperature (22−24 °C), controlled humidity (60−80%), and a 12-h light−dark cycle for 1 week before and during the experiments. The animals were allowed free access to standard laboratory chow and tap water. The test substance in four different doses (0.1, 1, 10, and 30 or 100 mg/kg) or vehicle (distilled water) alone was administered ip to groups of 10 mice on days 1, 3, and 5 with cyclophosphamide (CP) (30 mg/kg, po) administered on days 2, 4, and 6. One hour after the last immunosuppressant dose, the mice were challenged with a suspension of *C. albicans* sufficient to result in 70−90% mortality within 10 days in the vehicle (distilled water)-treated control group. In all experiments 1-[1-(2-cyano-1-aziridinyl)-1-methylethyl]-2-aziridinecarboxamide (azimexon)[43,50] was used as a control immunostimulating compound. A survival of 3 mice more in any group treated with test substance and CP than in the group treated with CP and distilled water was considered significant and possibly a result of immunorestorant activity.

2. In Vivo/in Vitro Tests. The in vivo/in vitro tests were performed on 3−4-week-old female HAN−NmRi strain mice, weighing 17−20 g. Before initiation of the experiment mice were veterinarily examined for any detectable disease. On the day of the experiment onset, 1 mL of Brewer's thioglycolate medium was administered to the animals by an ip injection. One hour later test substances were administered by the ip injection of 0.5 mL of a solution containing 25, 2.5, and 0.25 μg of compound **9** or MDP, respectively. In the same way the animals were treated on days 2 and 3. On day 4 the animals were sacrificed. Spleen was removed, and the peritoneal macrophages were washed out of the peritoneal cavity with ice-cold medium (RPMI 1640; Gibco, Great Britain).

Determination of the Number of Lymphocytes B and Lymphocytes T. For the determination of lymphocytes B, membrane-bound immunoglobulin G molecules on isolated splenic lymphocytes were marked with fluorescein isothiocyanate-labeled polyclonal goat anti-mouse Ig G antibodies (Sera-lab, Great Britain). For the determination of lymphocytes T, rat anti-Thy-1 monoclonal antibodies (Sera-lab, Great Britain) were used as primary antibodies and FITC-labeled polyclonal goat anti-rat Ig antibodies (Sera-lab, Great Britain) as the second antibody. After incubation and rinsing with RPMI 1640 medium, the lymphocytes were counted by fluorescence microscopy.

Determination of the Number of Macrophages. The number of macrophages in the peritoneal cavity washings was determined after trypan blue addition to the counting medium in Neubauer's blood cells counting glass chamber. Contaminating cells were excluded by morphology.

Blast Transformation of Lymphocytes by Mitogens.[27] Isolated spleen lymphocytes were prepared in the concentration of 1×10^6/mL in the nutrient RPMI 1640 medium (88 mL of RPMI 1640 supplemented with 10% fetal calf serum (FCS) (Sera-Lab, Great Britain), 1 mL of 200 mM l-glutamine solution, and 1 mL of an antibiotic solution (1×10^6 IU/mL penicillin and 100 μg/mL streptomycin)). To each flat bottom well of a microtitration plate (Nunc, Denmark) was distributed 100 μL of the cell suspension. The cells were stimulated in vitro by adding concanavalin A (Con A) (Pharmacia, Sweden) at respective concentrations of 16, 8, and 4 μg/mL. Control lymphocyte cultures were grown in nutrient RPMI 1640 medium only. The cells were then incubated at 37 °C in the presence of 5% CO_2 and at 95% humidity for 2 days followed by the addition of tritium-labeled thymidine (Amersham, Great Britain). After 16 h the samples were prepared for measurement in a β-counter (Beta Rach 1450, LKB Sweden). The results are expressed as incorporation indexes with respect to the control lymphocyte cultures.

Determination of Hemolytic Plaques for the Assessment of the Maturation of Lymphocytes B. A 1% suspension of sheep erythrocytes (Institute of Microbiology and Immunology, Ljubljana, Slovenia) in physiological saline was used for the ip immunization of mice. Individual mice were administered 0.2 mL of erythrocyte suspension followed the next day by 0.1 mL (1 μg/mouse) of the test substance. The immunization was completed on the fifth day when mice were sacrificed. Their spleens were removed and gently homogenized by squeezing between two sintered glass plates, and cells were suspended in a Parker 199 medium with added amino acids, streptomycin, and sodium carbonate. The lymphocytes were separated from other cells on Ficoll-Paque separating medium (Pharmacia, Uppsala, Sweden). After repeated rinsing with Parker 199 medium, the cells were resuspended in RPMI 1640 nutrient medium supplemented with 10% fetal calf serum and streptomycin (100 μg/mL). To 50 μL of the cell suspension was added 450 μL of the 1% trypan blue solution, and the cells were counted in Neubauer's chamber. The number of lymphocytes/mL of the cell suspension was calculated. To 100 μL of the diluted cell suspension were added 200 μL of RPMI 1640 nutrient medium, 50 μL of a 10% suspension of sheep erythrocytes, and 50 μL of guinea pig complement (Institute of Microbiology and Immunology, Ljubljana, Slovenia). Mixture of cells (MC) was pipetted into the testing chambers constructed on the glass slide. The chambers were sealed with white wax and cells incubated at 37 °C for 60 min. After completion of the incubation, the plaques were counted under a microscope. The number of plaques/1×10^6 cells was calculated according to the following equations:

$$\text{no. of plaques} = (1 \times 10^6 \text{ cells} \times \text{no. of plaques/chamber})/(\text{A cells/chamber})$$

$$\text{A cells/chamber} = [(\text{amount of MC/chamber } (\mu\text{L})) \times (\text{no. of cells in MC})]/[\text{amount of MC } (\mu\text{L})]$$

Activation of Peritoneal Macrophages. The peritoneal cavity of the animals was rinsed with 4 mL of ice-cold RPMI 1640 medium. The erythrocytes were lysed with 0.2% NaCl solution performing hypoosmotic shock for 1 min and followed by immediate reconstitution of the osmolarity with 1.2% NaCl solution. The remaining cells were washed twice in a cooled centrifuge at 4 °C and 1500 rpm for 5 min with ice-cold RPMI 1640 medium. The cells were resuspended, and the concentration was adjusted to 1.5×10^6/mL; 100 μL of the cell suspension was distributed to the flat bottom wells of a microtitration plate (Nunc, Denmark). After 2 h at 37 °C, 5% CO_2, and 95% humidity, the wells were rinsed with warm RPMI 1640 medium and the adhering macrophages were used for testing. The macrophages were covered either with 100 μL of a 160 μM ferricytochrome *c* solution in HBSS (Hank's balanced salt solution) without phenol red or with 100 μL of 160 μM ferricytochrome *c* and 200 nM phorbol myristate acetate (PMA; Sigma, St. Louis, MO) solution or with 100 μL of ferricytochrome *c*, PMA, and 340 U/mL superoxide dismutase (SOD; Sigma, St. Louis, MO) which specifically inhibited the reduction of cytochrome *c* with the superoxide ion (blank), respectively. After the samples were incubated for 90 min at 37 °C, 5% CO_2, and 95% humidity, the absorbance was measured at a wavelength of 570 nm.

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