Bioactive Pseudopeptidic Analogues and Cyclostereoisomers of Osteogenic Growth Peptide C-Terminal Pentapeptide, OGP(10−14)

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Received October 17, 2001

The osteogenic growth peptide (OGP) is a key factor in the mechanism of the systemic osteogenic response to local bone marrow injury. When administered *in vivo*, OGP stimulates osteogenesis and hematopoiesis. The C-terminal pentapeptide OGP(10−14) is the minimal amino acid sequence that retains the full OGP-like activity. Apparently, it is also the physiologic active form of OGP. Residues Tyr^{10}, Phe^{12}, Gly^{13}, and Gly^{14} of OGP are essential for the OGP(10−14) activity. The present study explored the functional role of the peptide bonds, carboxyl and amino terminal groups, and conformational freedom in OGP(10−14). Transformations replacing the peptide bonds with surrogates such as Ψ(CH_2NH), Ψ(CONMe), and Ψ(CH_2CH_2) demonstrated that amide bonds do not contribute significantly to OGP(10−14) bioactivity. End-to-end cyclization yielded the fully bioactive cyclic pentapeptide *c*(Tyr-Gly-Phe-Gly-Gly). The retroinverso analogue *c*(Gly-Gly-*phe*-Gly-*tyr*), a cyclostereoisomer of *c*(Tyr-Gly-Phe-Gly-Gly), is at least as potent as the parent cyclic pentapeptide. The unique structure−activity relations revealed in this study suggest that the spatial presentation of the Tyr and Phe side chains has a major role in the productive interaction of OGP(10−14) and its truncated and conformationally constrained analogues with their cognate cellular target.

Introduction

The osteogenic growth peptide (OGP) is a naturally occurring tetradecapeptide identical to the C-terminal amino acid sequence 89−102 (H-Ala-Leu-Lys-Arg-Gln-Gly-Arg-Thr-Leu-Tyr-Gly-Phe-Gly-Gly-OH) of histone H4 (H4).[1] The endogenous OGP is a proteolytic cleavage product of PreOGP translated from H4 mRNA via alternative translational initiation at a downstream initiation codon.[2] OGP in high abundance occurs physiologically in human and rodent serum and in serum-free medium of osteoblastic and fibroblastic cells. It is mainly found as an inactive complex with α_2-macroglobulin (α_2M). Following its dissociation from the complex with α_2M, the peptide is proteolytically cleaved, thus generating the C-terminal pentapeptide H-Tyr-Gly-Phe-Gly-Gly-OH [OGP(10−14)], which activates an intracellular G_i-protein−MAP kinase signaling pathway.[1−6]

Recently, OGP and OGP(10−14) have attracted considerable clinical interest. They increase bone formation and trabecular bone density and stimulate fracture healing when administered to mice and rats.[1,5,7,8] In cultures of osteoblastic and other bone marrow stromal cells derived from human and other mammalian species, OGP regulates proliferation, alkaline phosphatase activity, and matrix mineralization via an autoregulated feedback mechanism.[1,4,9] *In vivo* it also regulates the expression of type I collagen and the receptor for basic fibroblast growth factor.[8] In addition, OGP and OGP-(10−14) enhance hematopoiesis, including the stimulation of bone marrow transplant engraftment and hematopoietic regeneration after ablative chemotherapy.[10,11] Apparently, the hematopoietic effects of OGP and OGP-(10−14) are secondary to their effect on the bone marrow stroma.

A primary structure−activity analysis carried out recently has demonstrated that OGP(10−14) is the minimal OGP-derived sequence that retains the full OGP-like biological potency.[5] Elimination of the amino group at the N terminal in OGP(10−14) affords the desaminoTyr^{10}OGP(10−14), which retains ∼70% and ∼100% of the respective *in vitro* and *in vivo* potency of the full-length OGP. Because of the simplified structure, high *in vivo* potency, and the anticipated reduced enzymatic susceptibility of the desaminoTyr^{10}OGP(10−14), we selected this analogue as our lead peptide for detailed structural manipulations. These studies showed that the integrity of pharmacophores such as the Tyr and Phe side chains as well as the Gly residues at the C terminus is important for the optimal bioactivity of the OGP(10−14).[5]

Occasionally, a stepwise decrease in the peptidic nature of bioactive peptides by the replacement of peptide bonds with peptide bond surrogates or the introduction of global conformational constraint by end-to-end cyclization results in the enhancement of bioavailability and metabolic stability. Therefore, the present study explored the role of individual peptide

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10.1021/jm010479l CCC: $22.00
Published on Web 03/15/2002

bonds in the OGP(10−14) bioactivity, replacing them with peptide bond surrogates such as N-methylated ($CONCH_3$) and reduced (CH_2NH) amide bonds. In addition, we developed N-to-C-terminal cyclic analogues with reversed sequence and inverted chirality that displayed improved *in vivo* and *in vitro* activity.

Synthesis

The peptides reported herein were prepared by the solid-phase peptide synthesis methodology using *tert*-butyloxycarbonyl (Boc) chemistry and standard side chain protection strategy.[12] The N-terminal Boc group was removed at the end of each cycle with 30% trifluoroacetic acid (TFA) in dichloromethane (DCM). After neutralization with 10% *N*,*N*-diisopropylethylamine (DIEA) in DCM, the extension of the resin-bound peptide was carried out by the preformed symmetrical anhydride generated by *N*,*N*′-dicyclohexylcarbodiimide (DCC) in DCM.[13] Similarly, the last coupling in the desamino series, peptides **1**−**9**, employed the symmetrical anhydride generated from the 3-(4-hydroxyphenyl)propionic acid. Completion of the coupling reaction was monitored by the ninhydrin test.[14] Treatment of the fully assembled side chain protected, resin-bound peptide with anhydrous hydrogen fluoride containing 10% anisole as a cation scavenger simultaneously removed the side chain protecting groups and cleaved the peptide from the resin.[15]

Introduction of the $\Psi(CH_2NH)$ peptide bond isostere into analogues **5**−**8** and **10** was accomplished in the solid phase. The free N-terminal amino group of the resin-bound peptide was reductively alkylated by the requisite Boc-protected α-aminoaldehyde in the presence of sodium cyanoborohydride ($NaBH_3CN$) in DMF containing 1% AcOH. The aldehydes Boc-Tyr(OBzl)-H, Boc-Phe-H, and Boc-Gly-H were prepared by $LiAlH_4$ reduction of their corresponding *N*,*O*-dimethyl hydroxamates at 0−10 °C in dried THF.[16] All components were added at the same time to minimize racemization.[17]

Interestingly, in the early attempts to synthesize analogue **9**, we have obtained a hydrophobic product ($k' = 4.9$) that in the FAB-MS showed a protonated molecular ion ($[M + H]^+$) that is 338 atomic mass units (amu) higher than the anticipated pseudopeptide **9** (data not shown). This product corresponds to [desaminoTyr-Gly-$NHCH(CH_2\Phi)CH_2]_2$Gly-Gly-OH, suggesting that N-dialkylation of the nonhindered Gly by excess Boc-Phe-H took place during the reductive alkylation step and was elongated by the subsequent coupling cycles to produce a branched product.[18] Eventually, this reductive dialkylation was prevented by reducing the ratio of Boc-Phe-H to the free amino-terminus resin-bound Gly_2 to 1.2:1. Under these conditions the anticipated pseudopeptide **9** was obtained as the sole product. To avoid the dialkylation of the free amino-terminus resin-bound Gly during the reductive alkylation with Boc-Gly-H, the pseudopeptide **8** was prepared by esterification of Merrifield resin with the cesium salt of N-protected pseudopeptide Boc-Gly$\Psi(CH_2NZ)$Gly-OH by the standard procedure.[19]

The cyclization of linear peptides was carried out under optimized conditions (high dilution, 0 °C) with diphenylphosphoryl azide as the coupling agent.[20] Progress of the cyclization reaction was followed by HPLC, and the reaction was generally carried out for 2−3 days until the disappearance of the linear starting material. Under the above conditions only the cyclic monomers **11**−**13**, **15**−**20**, **22**, and **23** were obtained.

For the synthesis of the respective Aib- and Asp-containing cyclic pentapeptides **23** and **24**, we used Kaiser's oxime resin method.[21] Recently, a convenient method for the synthesis of cyclic peptides has been developed by the use of the *p*-nitrobenzophenone oxime resin.[22] The peptidyl oxime ester linkage to this resin, which has high acid stability, is highly reactive toward primary amines. The free amino terminus of the fully assembled oxime-resin-bound peptide interacts intramolecularly with the oxime ester moiety, leading to cyclization with a concomitant cleavage of the cyclic peptide from the resin. Cyclization on the resin takes advantage of the self-diluting effect, which favors unimolecular reactions over the formation of oligomeric byproducts via a bimolecular mechanism.[23] The cyclic OGP(10−14) analogues **23** and **24**, synthesized via on oxime resin cyclization, were obtained as the predominant products in the crude and were easily purified by reversed-phase high-performance liquid chromatography (RP-HPLC).

Purification of the synthetic peptides was accomplished in a single RP-HPLC step, yielding homogeneous products. The homogeneity of the peptides was established by TLC and analytical HPLC. Their structural integrity was confirmed by mass spectrometry and/or amino acid composition analysis (data not shown). The physicochemical data of synthetic OGP(10−14) analogues are detailed in Table 1.

Biological Evaluation

Previously, it has been demonstrated that the endogenous OGP C-terminal pentapeptide OGP(10−14) is a potent *in vitro* mitogen and *in vivo* stimulator of osteogenesis and hematopoiesis.[5,11] We also reported that the elimination of the α-amino function from the N-terminus is associated with a moderate reduction in the in vitro potency but not the *in vivo* potency,[5] which can be attributed to the potentially greater metabolic stability of the resultant desaminoTyr^{10}OGP(10−14) toward aminopeptidases.[24] In this study we therefore used the desaminoTyr^{10}OGP(10−14) as a platform to probe the contribution of the amide bonds to the mitogenic activity of OGP(10−14).

The activity was screened in osteoblastic MC3T3 E1 and fibroblastic NIH 3T3 cell cultures. In these cell systems, OGP and all of its active analogues so far tested (previously and herein), including those with low mitogenic activity, show a bell-shaped dose−response curve, which in the MC3T3 E1 and NIH 3T3 typically peaks at 10^{-13} and 10^{-11} M analogue concentration, respectively.[1,3−5] In addition, as previously reported,[5] the relative potencies of the different analogues showed a very high correlation between the osteoblastic and fibroblastic cell systems (Figure 1). We therefore consider the loss of activity of the presently reported, poorly potent analogues as authentic rather than representing a shift of the dose−response curve. Table 2 summarizes the activity of OGP(10−14) analogues with peptide bond surrogates. *N*-methylation [Ψ(CO−NMe)] of either Gly^{13} or Gly^{14} resulted in a dramatic loss of activity (analogues

Table 1. Physicochemical Characteristics of OGP(10−14) Analogues

analogue no.	analogue	HPLC (K'),[a] min	TLC[b] R_{f1}, min	TLC[b] R_{f2}, min	TLC[b] R_{f3}, min	FAB-MS (MH^+), m/z
1	desaminoTyr^{10},Sar^{11}OGP(10−14)	3.9 (A)	0.71	0.60	0.58	499.4
2	desaminoTyr^{10},*N*(Me)Phe^{12}OGP(10−14)	4.1 (A)	0.67	0.54	0.51	499.3
3	desaminoTyr^{10},Sar^{13}OGP(10−14)	3.5 (B)	0.71	0.60	0.66	499.4
4	desaminoTyr^{10},Sar^{14}OGP(10−14)	3.4 (C)	0.89	0.73	0.71	
5	desaminoTyr$^{10}\Psi(CH_2NH)$Gly^{11}OGP(10−14)	3.6 (A)	0.77	0.75	0.66	471.4
6	desaminoTyr^{10},Gly$^{11}\Psi(CH_2NH)$Phe^{12}OGP(10−14)	4.0 (A)	0.55	0.74	0.80	
7	desaminoTyr^{10},Phe$^{12}\Psi(CH_2NH)$Gly^{13}OGP(10−14)	3.6 (A)	0.82	0.57	0.77	471.6
8	desaminoTyr^{10}Gly$^{13}\Psi(CH_2NH)$Gly^{14}OGP(10−14)	4.5 (A)	0.81	0.76	0.79	471.5
9	desaminoTyr^{10}Gly$^{13}\Psi(CH_2CH_2)$Gly^{14}OGP(10−14)	2.4 (A)	0.60	0.81	0.80	
10	Tyr$^{10}(CH_2NH)$Gly^{11}OGP(10−14)	4.5 (D)	0.76	0.72	0.58	486.0
11	*c*(Tyr-Gly-Phe-Gly-Gly)	3.4 (A)	0.87	0.64	0.78	482.3
12	*c*(Gly-Gly-Phe-Gly-Tyr)	4.8 (A)	0.68	0.42	0.52	482.5
13	*c*(*tyr*-Gly-*phe*-Gly-Gly)	4.5 (A)	0.75	0.80	0.66	482.5
14	retro-inverso OGP(10−14)	2.8 (A)	0.66	0.49	0.10	500.0
15	*c*(Gly-Gly-*phe*-Gly-*tyr*)	4.8 (A)	0.60	0.35	0.24	482.5
16	*c*(Tyr-Gly-Phe-Gly)	3.0 (A)	0.53	0.52	0.48	
17	*c*(Gly-Tyr-Gly-Phe-Gly-Gly)	3.8 (A)	0.75	0.65	0.86	
18	*c*(*β*Ala-Tyr-Gly-Phe-Gly-Gly)	4.0 (A)	0.64	0.54	0.66	553.2
19	*c*(*γ*Abu-Tyr-Gly-Phe-Gly-Gly)	4.8 (A)	0.73	0.73	0.78	567.5
20	*c*(*δ*Ava-Tyr-Gly-Phe-Gly-Gly)	5.0 (A)	0.36	0.42	0.90	581.2
21	[Pro^{11}]OGP(10−14)	2.8 (A)	0.40	0.53	0.55	
22	*c*(Tyr-Pro-Phe-Gly-Gly)	4.6 (A)	0.79	0.75	0.55	522.0
23	*c*(Tyr-Aib-Phe-Gly-Gly)	4.9 (C)	0.83	0.79	0.66	
24	*c*(Tyr-Gly-Phe-Gly-Asp)-OH	3.5 (A)	0.75	0.69	0.36	540.5

[a] Linear acetonitrile gradient (30 min) used: A, 15−40%; B, 5−95%; C, 5−25%; D, 5−35%. [b] Solvent system used (v/v): (1) 1-BuOH/AcOH/H_2O (4:1:1); (2) 1-BuOH/AcOH/EtOAc/H_2O (5:1:3:1); (3) $CHCl_3$/MeOH/AcOH (9:3:1).

Table 2. Proliferative Activity of Pseudopeptide OGP(10−14) Analogues with Peptide Bond Surrogates

analogue no.	analogue	relative potency (95% confidence limit) MC 3T3 E1 cells	relative potency (95% confidence limit) NH3T3 cells
	OGP(10−14)	1.00 (reference)	1.00 (reference)
1	desaminoTyr^{10},Sar^{11}OGP(10−14)	0.31 (0.25−0.37)	0.39 (0.26−0.52)
2	desaminoTyr^{10},*N*(Me)Phe^{12}OGP(10−14)	0.52 (0.46−0.58)	0.67 (0.55−0.70)
3	desaminoTyr^{10},Sar^{13}OGP(10−14)	0.15 (0.07−0.23)	0.11 (0.05−0.17)
4	desaminoTyr^{10},Sar^{14}OGP(10−14)	0.16 (0.10−0.22)	0.14 (0.09−0.19)
5	desaminoTyr$^{10}\Psi(CH_2NH)$Gly^{11}OGP(10−14)	0.81 (0.71−0.91)	0.79 (0.67−0.91)
6	desaminoTyr^{10},Gly$^{11}\Psi(CH_2NH)$Phe^{12}OGP(10−14)	0.61 (0.53−0.69)	0.67 (0.60−0.74)
7	desaminoTyr^{10},Phe$^{12}\Psi(CH_2NH)$Gly^{13}OGP(10−14)	0.70 (0.65−0.75)	0.88 (0.76−1.00)
8	desaminoTyr^{10}Gly$^{13}\Psi(CH_2NH)$Gly^{14}OGP(10−14)	0.78 (0.73−0.83)	0.80 (0.67−0.93)
9	desaminoTyr^{10}Gly$^{13}\Psi(CH_2CH_2)$Gly^{14}OGP(10−14)	0.78 (0.73−0.83)	0.88 (0.79−0.97)
10	Tyr$^{10}\Psi(CH_2NH)$Gly^{11}OGP(10−14)	0.92 (0.83−1.01)	0.87 (0.83−0.91)

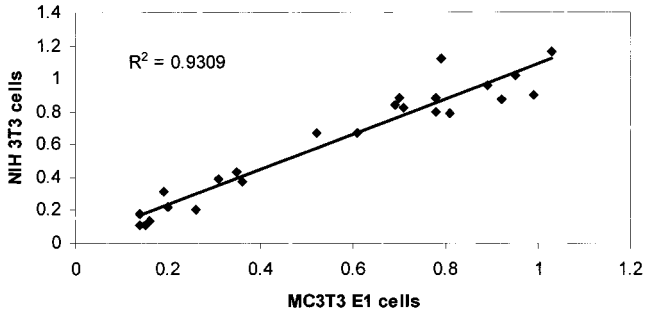

Figure 1. Regression analysis of proliferative potency of pseudopeptide and cyclic OGP(10−14) analogues between osteoblastic MC3T3 E1 and fibroblastic NIH 3T3 cell lines. Values are *in vitro* potencies relative to activity of OGP-(10−14).

3 and **4**). This modification is more acceptable at position 12 (analogue **2**) than position 11 (analogue **1**), with the former being almost 2-fold more potent than the latter. Interestingly, all amide bonds are equally more tolerant to amide bond reduction [$\Psi(CH_2NH)$] than to N-methylation (cf. analogues **1**−**4** and **5**−**8**, Table 2). As in the case of the parent pentapeptide, the Tyr$^{10}\Psi(CH_2NH)$Gly^{11}-containing pentapseudopeptide is more potent than the corresponding desamino derivative (cf. analogues **10** and **5**). Apparently, the similar potency of analogues **8** and **9**, containing a $\Psi(CH_2NH)$ and a $\Psi(CH_2CH_2)$ between Gly^{13} and Gly^{14}, suggests that the chemical nature of this bond is of minor significance. While *N*-methylation of the amide bond reduces the barrier for rotation around the modified bond and therefore increases the population of the *cis* vs *trans* isomer,[25] the reduction and "carba" modifications of the amide bond increase local flexibility at the site of the peptide bond surrogate.[26] Thus, the lower potency of the N-methylated amide bond series compared to the parent peptide and reduced amide bond series may be related to the steric hindrance introduced by the N−CH_3 group. At this stage we cannot determine whether this modification perturbs the bioactive conformation or the fully productive interaction of the bioactive conformation with the putative receptor.

Structural rigidification provides the means to delineate the conformational space and offers insights into the biologically relevant conformations. In general, end-to-end cyclization, especially when carried out on small peptides, is an established mode for introducing global

Table 3. Proliferative Activity of Cyclostereoisomers of OGP(10−14)

analogue no.	analogue	relative potency (95% confidence limit) MC 3T3 E1 cells	relative potency (95% confidence limit) NH3T3 cells
	OGP(10−14)	1.00 (reference)	1.00 (reference)
11	*c*(Tyr-Gly-Phe-Gly-Gly)	0.79 (0.72−0.86)	1.12 (1.06−1.17)
12	*c*(Gly-Gly-Phe-Gly-Tyr)	0.95 (0.89−1.01)	1.02 (0.93−1.11)
31	*c*(D-Tyr-Gly-D-Phe-Gly-Gly)	0.69 (0.62−0.76)	0.84 (0.80−0.88)
14	*retro-inverso* OGP(10−14)	0.71 (0.52−0.90)	0.82 (0.75−0.89)
15	*c*(Gly-Gly-phe-Gly tyr)	1.03 (0.95−1.11)	1.16 (1.10−1.22)

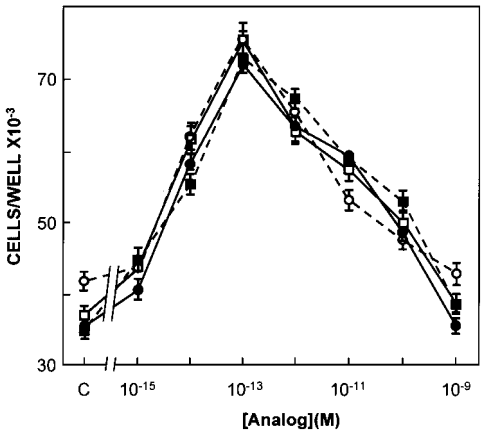

Figure 2. Cyclostereoisomers of OGP(10−14) retain full OGP-like, dose-dependent proliferative activity in osteoblastic MC3T3 E1 cell culture: (○) OGP(10−14); (●) *c*(Tyr-Gly-Phe-Gly-Gly); (□) *c*(Gly-Gly-Phe-Gly-Tyr); (■) *c*(Gly-Gly-*phe*-Gly-*tyr*). Data are mean ± SE obtained in triplicate culture wells per condition.

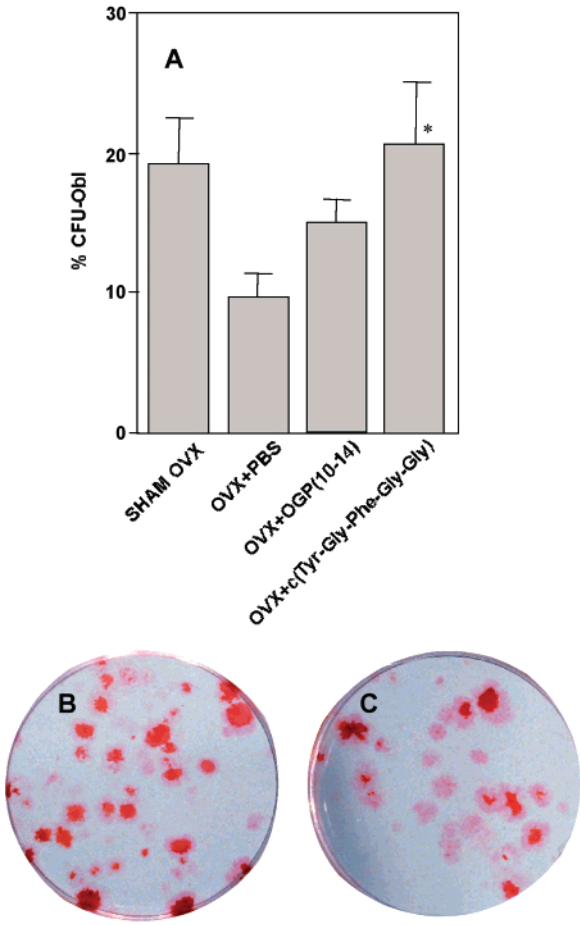

Figure 3. *c*(Tyr-Gly-Phe-Gly-Gly) rescues CFU-Obl derived from femoral and tibial bone marrow of OVX rats. (A) Shown is the percent CFU-Obl of total CFU-f in sham-OVX, untreated control rats, and OVX rats treated with either PBS vehicle or indicated peptides. Data are mean ± SE obtained in five rats per condition. (B, C) double ALP/ARS staining of CFU-f derived from OVX rats treated with OGP(10−14) (B) or PBS (C). Note the absence of single-positive ARS colonies.

rigidification.[27] Remarkably, all four cyclostereoisomers of the C-terminal OGP-derived pentapeptide, the *all*-L-isomer (**11**), the *retro*-isomer (**12**), the *all*-D-isomer (**13**), and the *retro-inverso* isomer (**15**), were highly mitogenic (Table 3), demonstrating a dose/response relationship identical to that of the parent OGP(10−14) (Figure 2). The cyclic *retro-inverso* pentapeptide **15** was the most active cyclostereoisomer, significantly more active than the parent linear peptide OGP(10−14) and substantially more potent than the linear *retro-inverso* pentapeptide **14** (Table 3). These results suggest that the charged amino and carboxyl end groups and the nonpalindromicity of the backbone do not have an important role in the interaction with the putative OGP receptor. Moreover, global conformational constraint is not detrimental to bioactivity. Comparison of the potencies of the enantiomeric pairs **11** and **13**, and **12** and **15** indicates opposite effects of chain reversal and chirality switch. The *all*-L-isomer **11** is more potent than its enantiomer **13** (same chain direction but opposite chirality), and the *retro-inverso* isomer **15** (reversed chain direction and opposite chirality) is more active than the *retro*-isomer **12** (reversed chain direction and the same chirality). Formally, the *retro-inverso* analogue **15**, the most reactive cyclostereoisomer, is topologically equivalent to the *all*-L-isomer **11**.

In vivo, even the *all*-L-cycloisomer (**11**) was more effective than the less rigid linear OGP(10−14) in reversing the ovariectomy (OVX)-induced decrease in colony-forming units, osteoblastic (CFU-Obl) (Figure 3). *Ex vivo* CFU-Obl is a surrogate of the *in vivo* number of preosteoblastic cells capable of terminal osteoblastic differentiation and bone formation.[28] The stimulation of CFU-Obl by OGP(10−14) and its *all*-L-cycloisomer is in line with our previous demonstration of anabolic activity in the bone of the full-length OGP, OGP(10−14), and desaminoTyr[10]OGP(10−14).[1, 5] The CFU-Obl stain positively for both alkaline phosphatase (ALP) and alizarin-red-S (ARS, staining of mineral deposits). Because mineralization is the final stage of osteogenic differentiation preceded by the expression and activity of ALP,[29] ARS positive deposits typically occur only in the center of ALP positive colonies (Figure 3B,C). In this context it should be pointed out that the OGP(10−14) and its *all*-L-cycloisomer affected only the number of the double-positive ALP/ARS colonies and not the number of total or single-positive ALP CFUs (data not shown). It is therefore suggested that in the bone marrow system, OGP(10−14) and its cyclic analogues preferen-

Table 4. Proliferative Activity of Variable Ring Size Cyclic OGP(10−14) Analogues

analogue no.	analogue	relative potency (95% confidence limit) MC 3T3 E1 cells	relative potency (95% confidence limit) NH3T3 cells
	OGP(10−14)	1.00 (reference)	1.00 (reference)
16	*c*(Tyr-Gly-Phe-Gly)	0.35 (0.30−0.40)	0.43 (0.40−0.46)
17	*c*(Gly-Tyr-Gly-Phe-Gly-Gly)	0.26 (0.19−0.33)	0.20 (0.17−0.23)
81	*c*(βAla-Tyr-Gly-Phe-Gly-Gly)	0.36 (0.30−0.42)	0.37 (0.31−0.43)
19	*c*(γAbu-Tyr-Gly-Phe-Gly-Gly)	0.20 (0.16−0.24)	0.22 (0.19−0.25)
20	*c*(δAva-Tyr-Gly-Phe-Gly-Gly)	0.14 (0.09−0.19)	0.18 (0.13−0.23)

Table 5. Proliferative Activity of Miscellaneous Cyclic OGP(10−14) Analogues

analogue no.	analogue	relative potency (95% confidence limit) MC 3T3 E1 cells	relative potency (95% confidence limit) NIH3T3 cells
	OGP(10−14)	1.00 (reference)	1.00 (reference)
21	[Pro^{11}]OGP(10−14)	0.89 (0.80−0.98)	0.96 (0.87−1.05)
22	*c*(Tyr-Pro-Phe-Gly-Gly)	0.99 (0.79−1.19)	0.90 (0.77−1.03)
23	*c*(Tyr-Aib-Phe-Gly-Gly)	0.19 (0.14−0.24)	0.31 (0.29−0.33)
24	*c*(Tyr-Gly-Phe-Gly-Asp)-OH	0.14 (0.09−0.19)	0.11 (0.07−0.15)

tially stimulate cells in their late stage of osteoblastic differentiation.

Contraction (analogue **16**, a 12-member ring) or expansion (analogues **17**−**20**, 18- to 21-member rings, respectively) of the ring size of the cyclopentapeptide **11** (a 15-member ring) leads to substantial loss in proliferative potency (Table 4). Evidently, over-rigidification, as in the case of the cyclotetrapeptide **16**, results in 2- to 3-fold reduction in potency compared to the cyclopentapeptide **11**. It may suggest that the contraction in ring size eliminates some degree of conformational freedom, which is apparently important for the formation of the putative bioactive conformation. An even greater loss of activity results from increasing the degree of conformational freedom by adding, between the ring-forming end groups, a Gly residue or homologues thereof as spacers, thus generating homologous cyclohexapeptides that are 2- to 7-fold less potent than the linear parent pentapeptide and the cyclopentapeptide **11**. These results suggest that the cyclohexapeptides **17**−**20**, which are conformationally constrained in comparison to the flexible linear pentapeptide, not only do not favor the putative bioactive conformation but may also discriminate against it.

Local rigidification between Tyr^{10} and Phe^{13} was achieved by replacing Gly^{12} with either Pro or Aib (Table 5). The former represents an intraresidue side-chain-to-backbone rigidification, while the latter represents a rigidification that originates from steric hindrance contributed by the disubstituted C_α carbon. Interestingly, position 12 accommodates substitution by Pro, both when introduced to the linear pentapeptide **21** or to the cyclopentapeptide **22**. However, introduction of Aib^{11} results in a 4- to 10-fold loss of potency. It may be speculated that the different conformational biases of Pro and Aib may lead to differential effects on the bioactive conformation. For example, Aib, which is known to promote helical conformation,[30] is not tolerated, while Pro, which is a helix breaker,[31] is well tolerated.

Earlier studies carried out in our laboratories suggested that the C-terminal free carboxyl is crucial for mitogenic activity. Amidation, esterification, and reduction of this carboxyl led to a 2- to 30-fold loss in potency.[5] We revisited the role of the carboxyl function in the context of the cyclic C-terminal pentapeptide, in which this function was "sacrificed" during the end-to-end cyclization. To this end, we prepared the cyclic pentapeptide **24** in which Gly^{14} is replaced with Asp, thus yielding a carboxyl-bearing side chain, which mimics the original C-terminal carboxyl in the linear parent pentapeptide (Table 5). The 5- to 10-fold decrease in potency of analogue **24** compared to the parent cyclopentapeptide **11** suggests that the presentation of the carboxyl in the form of Asp^{14} is compromising the interaction with the putative receptor. It cannot be ruled out that introduction of Asp^{14} presents the carboxyl in an unfavorable spatial orientation for taking advantage of a putative complementary charge within the receptor.

Conclusion

Our studies of the OGP structure−activity relationship led to the identification of the C-terminal pentapeptide, H-Tyr-Gly-Phe-Gly-Gly-OH, as the minimal sequence required for full biological activity. In this sequence, the Tyr and Phe side chains act as the critical pharmacophores. The promiscuity of the peptidic and nonpalindromic nature of the backbone allowed for the development of the *retro-inverso* cyclic pentapeptide, *c*(Gly-Gly-*phe*-Gly-*tyr*), a fully potent cyclostereoisomer of the *c*(Tyr-Gly-Phe-Gly-Gly), which is equipotent to the linear OGP(10−14). These findings suggest that the molecular topology in general and the spatial disposition of the pharmacophores in particular play a key role in the recognition and activation of the putative macromolecular OGP receptor.

Experimental Section

General. Boc-amino acids[32] were purchased from either Bachem California or prepared with di-*tert*-butyl dicarbonate by a conventional procedure.[33] All chemicals were purchased from either Aldrich Chemica Co., Fluka Chemie AG Switzerland, or Pierce Chemical Co. and were used without further purification. Where appropriate, solvents were freshly distilled from proper drying materials before use. The tyrosine side chain was protected as an *O*-benzyl ether, namely, Boc-Tyr-(O-Bzl)-OH. Peptides were treated with liquid HF in an all-Teflon apparatus (Protein Research Foundation, Osaka, Japan). Thin-layer chromatography (TLC) was performed on precoated silica gel plates 60F-254 (E. Merck, Darmstadt, FRG) in the following solvent systems (all v/v): (1) 1-BuOH/AcOH/H_2O (4:1:1); (2) 1-BuOH/AcOH/EtOAc/H_2O (5:1:3:1); (3) $CHCl_3$/MeOH/AcOH (9:3:1). Peptides were visualized by one

or two of the following procedures: (1) UV; (2) ninhydrin spray reagent. Analytical and semipreparative HPLC separations were performed on a Merck Hitachi 655A-11 liquid chromatograph, equipped with a model 655A variable wavelength and model L-5000 LC controller, model D-2000 chromatointegrator, and a model AS-2000 autosampler injector. Absorptions were measured at 220 nm. For all analytical applications, a Lichrospher 100 RP-18 column was used. The crude peptides were purified on a μBondapak C-18, 19 cm × 150 cm column or a Vydac Protein & Peptide C-18 column. HPLC was carried out using a gradient made from two solvents: (A) 0.1% TFA in water and (B) 0.1% TFA in acetonitrile. The gradient conditions used for purification were determined by analytical HPLC. The flow rates used for the analytical and semipreparative columns were 1 and 6 mL/min, respectively. The analytical column was protected with a C-18 guard cartridge. The crude peptides were dissolved in appropriate solvents and then filtered through a PTFE filter (0.2 mm) and directly injected. In some cases, a repeated purification step was required to obtain high purity. Fractions containing the purified peptides were pooled and lyophilized.

Peptide Synthesis. (A) Synthesis of Linear Peptides, General. The OGP analogues were synthesized on a Milligen 504 synthesizer (manual) or a 430A Applied Biosystem peptide synthesizer unless otherwise indicated. Boc-amino acids were assembled on a PAM resin or Merrifield resin. The PAM resin was purchased with the first Boc-amino acid already attached. The Boc-amino acid-resin was subjected to the required number of coupling and deprotection cycles according to the SPPS method of choice. In each cycle, resins were treated with 25% TFA in the presence of 1% dimethyl sulfide (DMS) in DCM to remove Boc groups. After neutralization with 10% DIEA in DCM, the resin was treated with a 4-fold excess of the appropriate DCC-mediated preactivation of the Boc-amino acids in DCM. Where possible, completion of each coupling step was monitored by the Kaiser test. The fully assembled peptides were removed from the resin either by aminolysis (in the case of Merrifield resin-bound peptide) or by treatment with anhydrous hydrogen fluoride (HF, 18 mL/g resin-bound peptide) containing anisole (2 mL/g resin-bound peptide) as a cation scavenger at −5 °C for 1 h. After the removal of HF *in vacuo*, the residues were precipitated with diethyl ether and extracted from the resin with 30% aqueous acetic acid. The aqueous fractions were combined and then lyophilized.

The peptides were assayed for purity using analytical HPLC (Vydac C-18 column) and were shown to be >95% pure. Peptides were hydrolyzed for amino acid analysis in 6 N HCl for 24 h at 110 °C in vacuum-sealed tubes. The samples were analyzed after precolumn derivatization as Fmoc-amino acid, performed at the Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, Israel. Molecular weights of the peptides were determined by FAB-MS at the Faculty of Chemistry, Technion, Haifa, Israel.

Boc−*N*-Me−Phe−OH. The preparation followed a previously reported procedure.[34] Briefly, Boc-Phe-OH was dissolved in dry THF and treated with MeI (8 equiv). NaH (60% dispersion in oil, 3 equiv) was added portionwise to the reaction mixture, and the reaction was monitored by HPLC. The solvent was removed *in vacuo*, yielding a crude product that was purified by flash column chromatography eluted with EtOAc/petroleum ether to yield the pure product.

DesaminoTyr$^{10}\Psi$[CH$_2$NH]Gly^{11}-Phe-Gly-Gly-OH (5). 3-(4-hydroxyphenyl)propionic acid (1.33 g, 8.0 mmol) was dissolved in dry DCM (50 mL), and the salt of *N*,*O*-dimethylhydroxylamine (0.9 g, 9.2 mmol), Et_3N (1.22 mL, 8.8 mmol), DCC (1.65 g, 8.0 mmol), and *p*-(dimethylamino)pyridine (DMAP) (0.0486 g, 3.9 mmol) were added. The solution was stirred overnight at room temperature, and then the DCCU was filtered off and the solvents were evaporated to dryness. The residue was dissolved in EtOAc and was washed with a saturated aqueous solution of NaCl (2 × 20 mL), a saturated aqueous solution of $NaHCO_3$ (2 × 20 mL), a 10% aqueous solution of citric acid (2 × 20 mL), and finally a saturated aqueous solution of NaCl. The organic layer was dried over anhydrous $MgSO_4$ and was evaporated to dryness. The crude product was purified by column chromatography on silica gel eluted with EtOAc/petroleum ether, obtaining 1.03 g (62%) of HO−$C_6H_5CH_2CH_2$-CON(OMe)Me as an oil.

HO−$C_6H_5CH_2CH_2$CON(OMe)Me (0.24 g, 1.44 mmol) was dissolved in anhydrous diethyl ether, and the solution was cooled to 0 °C, and then 0.069 g (1.8 mmol) of $LiAlH_4$ was added in small portions. After 3 h, 3 mL of a 10% aqueous solution of $KHSO_4$ was added dropwise and the aqueous layer was extracted with diethyl ether (3 × 30 mL). The organic layer was collected and washed with 3 N HCl (2 × 20 mL), a saturated solution of $NaHCO_3$ (2 × 20 mL), and a saturated solution of NaCl (2 × 20 mL). The solution was then dried over anhydrous $MgSO_4$ and was evaporated to dryness to obtain 0.17 g (77%) of 4-HO−$C_6H_5CH_2CH_2CHO$, which was used without further purification.

3-(4-Hydroxyphenyl)propional (50 mg, 0.33 mmol) was dissolved in dry DMF (10 mL) containing 1% AcOH, and the solution was added to H-Gly-Phe-Gly-Gly-PAM resin (0.3 mmol, prepared by SPPS) followed by the addition of $NaCNBH_3$ (0.4 mmol) in several portions. The reaction mixture was allowed to shake overnight at room temperature followed by removal of the N-terminal protecting group with 25% TFA in DCM and a liquid HF/anisole cleavage step. The crude peptide (84 mg) was purified on preparative HPLC to yield the pure peptide (8 mg).

DesaminoTyr-Gly$^{11}\Psi$(CH$_2$NH)Phe^{12}-Gly-Gly-OH (6). The *N*,*O*-dimethyl hydroxamate of Boc-Gly-OH was obtained as a white solid from the DCC coupling of Boc-Gly-OH with *N*,*O*-dimethylhydroxylamine in the presence of DMAP and then was converted to Boc-glycinal by $LiAlH_4$ reduction. The peptide resin Phe-Gly-Gly-PAM was reductively alkylated with Boc-glycinal, as above, and then elongated by coupling with 3(4-hydroxyphenyl)propionic acid followed by cleavage of the peptide from the resin with HF/anisole. The crude peptide was purified using semipreparative HPLC with 0−60% acetonitrile in 120 min, thus affording the pure peptide.

DesaminoTyr-Gly-Phe$^{12}\Psi$(CH$_2$NH)Gly^{13}-Gly-OH (7). The *N*,*O*-dimethyl hydroxamate of Boc-Phe-OH was obtained as a colorless oil from the BOP-mediated coupling of Boc-Phe-OH with *N*,*O*-dimethylhydroxylamine in the presence of triethylamine. Reduction of the *N*,*O*-dimethyl hydroxate of Boc-Phe-OH with $LiAlH_4$ afforded Boc-phenylalaninal (Boc-Phe-H) as a white solid, which was used without further purification. A total of 1.25 equiv of the crude aldehyde was dissolved in dry DMF (15 mL) containing 1% AcOH, and the solution was added to H-Gly-Gly-PAM resin (0.4 mmol), which had been prepared by the SPPS procedure described above. This was followed by the addition of $NaCNBH_3$ (0.5 mmol) in several portions. The reaction mixture was allowed to shake overnight, and then *Z*-Cl and TEA (5 equiv) were added to protect the secondary amine between Phe and Gly. The reaction was carried out overnight and the N-terminal Boc group was removed by 25% TFA in DCM. The peptide was further elongated by coupling sequentially with Boc-Gly and 3-(4-hydroxyphenyl)propionic acid, followed by cleavage from the resin with liquid HF/anisole. The crude peptide was purified using semipreparative HPLC with 0−60% acetonitrile in 120 min, which afforded the desired peptide.

DesaminoTyr-Gly-Phe-Gly$^{13}\Psi$(CH$_2$NH)Gly^{14}-OH (8).

Boc-NHCH$_2$CH$_2$NH$_2$ (Boc-EDA). A solution of di-*tert*-butyl dicarbonate (4.3 g, 19.7 mmol) in DCM (70 mL) was added dropwise (5 mL/min) to a solution of 1,2-diaminoethane (13 mL, 195 mmol) in DCM (70 mL). The reaction mixture was then left to stir for 24 h at room temperature, followed by extraction with cold 1 N $KHSO_4$ (3 × 20 mL). The aqueous phase was pooled together, and solid NaOH was added until pH 10−11 was reached. The alkaline aqueous solution was extracted with DCM (3 × 20 mL), which was pooled together and dried over $MgSO_4$. Evaporation under reduced pressure yielded Boc-EDA as an oil (1.52 g, 48.3%).

Boc-GlyΨ(CH$_2$NH)Gly-OH. A solution of chloroacetic acid (2.3 g, 24.3 mmol) in aqueous NaOH (1 N, 24.4 mL) was added dropwise to a solution of Boc-EDA (2.46 g, 15.3 mmol) in

ethanol (95%, 6 mL) followed by addition of 1 N NaOH (12.2 mL). The reaction mixture was stirred overnight at room temperature, and the ethanol was removed *in vacuo*. The residue was extracted with diethyl ether (3 × 20 mL). The aqueous phase was cooled to 0 °C, and benzyloxycarbonyl chloride (1.9 g) was added dropwise, maintaining pH 9−10 with 1 N NaOH. After being stirred for 5 h at room temperature, the reaction mixture was extracted with diethyl ether, cooled to 0 °C, acidified with cold citric acid solution (30%), and washed with EtOAc. Evaporation of the washed and dried EtOAc extract resulted in an oil that was recrystallized on trituration with petroleum ether to yield 2.3 g (42.6%).

The Boc-protected pseudodipeptide (0.7 g, 2 mmol) was attached to Merrifield resin using the cesium salt. The resin-bound peptide was treated with 25% TFA in DCM, neutralized with DIEA, and then elongated with Boc-Phe-OH, Boc-Gly-OH and 3-(4-hydroxyphenyl)propionic acid. After cleavage from the resin, the crude peptide was purified by preparative HPLC to yield the pure product.

(B) Cyclic Analogues. Method 1. Solution Cyclization. The crude linear peptides were purified on preparative HPLC, or the nonpurified peptide was used directly for cyclization. Solid $NaHCO_3$ (5 equiv) was added to a cooled (0 °C) solution of the linear peptide in an anhydrous, amine-free DMF (0.008 M), and the suspension was stirred for 5 min. After addition of diphenylphosphoryl azide (DPPA) (1.5 equiv), the reaction mixture was stirred at 0−5 °C for 2−4 days and HPLC-monitored. Upon completion of the reaction, the solvent was evaporated to dryness *in vacuo* (30 °C bath). The residues were purified on preparative HPLC to yield the pure cyclic peptides.

Method 2. On-Resin Cyclization Employing Oxime Resin. Boc-amino acids and DCC (1 equiv) were added to a suspension of the oxime resin (2 equiv) in DCM in a shaking reaction vessel. After 15 h, the solvent was drained and the resin was washed with DCM, DCM/EtOH (1:1), and DCM. Excess oxime functional groups were capped with AcOH (1.5 equiv) and DIEA (1.5 equiv) in DCM. The Boc-amino acyl-oxime resin was obtained after washing with DCM, EtOH/DCM (1:1), EtOH, and DCM and was dried in vacuo. The level of resin substitution was determined with picric acid. Single couplings were performed with 3 equiv of a suitable protected amino acid activated by BOP. A typical cycle for the coupling of an individual amino acid was as follows: (i) activation of the next amino acid to be coupled with BOP; (ii) deprotection of the amino acid on the resin with 25% TFA/DCM + 1% DMS as a scavenger; (iii) washing with DCM and neutralization with 10% DIEA/DCM; (iv) mixing the BOP-activated amino acid with the neutralized resin-bound peptide. The efficiency of each coupling was monitored by the Kaiser test.

After final removal of the Boc protecting group from the N terminus, the amino group was liberated from its TFA salt by addition of DIEA (1.5 equiv). Intrachain aminolysis by the free amino group cleaved the peptide from the oxime resin, generating concomitantly the cyclic peptide. These cleavage/cyclization reactions were carried out in DCM at room temperature and catalyzed by the presence of 1.5 equiv of AcOH.[35] After 24 h of reaction time, the cyclic peptides were obtained from the solution phase by filtration and purified by preparative HPLC to yield a pure cyclic peptide.

Deprotection Procedure. Catalytic hydrogenation was carried out in a solution mixture of 10% AcOH/MeOH under atmospheric pressure at room temperature in the presence of 5% Pd black using a peptide-to-catalyst ratio of 3:1. After complete removal of the benzyl-type protecting groups as monitored by analytical HPLC, the solution was filtered, the filtrate concentrated *in vacuo*, and the residue subjected to purification on preparative HPLC.

Peptide purification was evaluated using analytical HPLC (Vydac C-18 column) and was shown to be >95% in all cases. Peptides were hydrolyzed for amino acid analysis in 6 N HCl for 24 h at 110 °C in deaerated tubes. The samples were analyzed after precolumn derivatization as the Fmoc-amino acid. Molecular weights of the peptides were determined by FAB-MS.

***In Vitro* Proliferation Assay.** This assay was carried out as reported recently.[5] Briefly, osteoblastic MC3T3 E1 and fibroblastic NIH 3T3 cells derived from confluent maintenance cultures grown in minimal essential medium (αMEM) supplemented with 10% fetal calf serum (FCS) were seeded in 16 mm multiwell dishes at 2×10^4 cells per well and incubated in the same medium at 37 °C in CO_2/air. For the initial 46 h the cells were incubated in the same FCS-containing medium. The cultures were then washed and kept for an additional 2 h period under serum-free conditions. This was followed by replacement of the serum-free αMEM with a medium containing 4% fatty acid free bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO; catalog no. A-7030) with or without OGP(10−14) (positive control) or its analogue preparations. The analogue-containing culture medium was prepared as follows: the corresponding OGP(10−14) analogue [or OGP(10−14)] was freshly weighed, dissolved in the BSA-supplemented medium at 10^{-6} M, and further diluted to the final concentration with the same medium. Prior to use in the cell culture, the BSA-supplemented αMEM with the final analogue concentration was preincubated for 30 min at 37 °C. Unless otherwise specified, the analogue concentration was 10^{-13} and 10^{-11} M in the MC3T3 E1 and NIH 3T3 cell culture, respectively. By use of a variety of OGP and OGP(10−14) analogues, these concentrations were shown previously to induce maximal proliferative activity in the corresponding cell cultures.[3] To verify the relevance of these concentrations for the present study, some of the analogues were subjected to a dose-response analysis (Figure 2). Cell counts were carried out using a hemocytometer 48 h after addition of the BSA supplemented, analogue-containing αMEM.

The results were initially expressed as the mean cell number in triplicate culture wells per condition. The value obtained was then transformed into peptide-treated over peptide-free control ratios (T/C ratio) and then presented as relative potency using results obtained in cultures challenged with OGP(10−14) as reference. As in previous studies of the same nature,[36] the relative potencies of individual analogues in each of the cell lines were expressed as the mean and 95% confidence limit obtained in at least four repetitive experiments.

Reversal of OVX-Induced Reduction in CFU-Obl. Fifteen female Sabra rats (The Hebrew University animal facility, Jerusalem), each weighing 250 g, were subjected to bilateral OVX. Additional five control animals underwent sham OVX. All animals were left untreated for 30 days. Then the OVX animals were divided into three groups of five rats each that were treated daily for 8 weeks by subcutaneous injections of 100 ng/day/rat of either OGP(10−14) or *c*(Tyr-Gly-Phe-Gly-Gly) or the peptide-free PBS vehicle. The animals were sacrificed 1 day after treatment termination by an anesthetic overdose (Avertin, 400 mg/Kg). Prior to death, bone marrow from the two femoral and two tibial diaphyses of each animal was flushed into tissue culture medium (αMEM) and pooled. Determination of stromal colony forming units, fibroblastic (CFU-f, which include the CFU-Obl), was set as described previously.[28] Briefly, a suspension of single cells was prepared by withdrawing and expelling the bone marrow through graded syringe needles. The cells were seeded in 35 mm dishes at 10^6 cells/dish, 10 dishes per animal using αMEM supplemented with 50 mg/mL ascorbic acid, 10 mM β-glycerophosphate, and 10% fetal calf serum; these supplements promote osteoblastic differentiation and formation of mineralized nodules.[29] The medium was changed twice a week.

On day 21 in culture, the CFU-f were counted and then washed with PBS, fixed with citrate/acetone/formalin, and double-stained for ALP (Sigma 86-R kit) and mineral deposits (1% aqueous ARS). Counts of ALP positive colonies and double-positive ALP/ARS colonies (representing CFU-Obl) then followed. A colony was defined as a cluster of cells containing 16 or more members. The scores from each 10 dishes were averaged for the individual animals. Results were expressed as percent ALP positive CFU or percent CFU-Obl of the total CFU-f.

Acknowledgment. This study was supported by Grant No. 91-270 from the United States−Israel Binational Science Foundation and Grant No. 6305194 from the Ministry of Science and The Arts, The Government of Israel. Y.C. was a postdoctoral research fellow of The Hebrew University of Jerusalem.

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JM010479L