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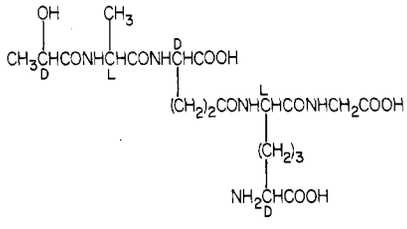
Communications to the Editor

N^2-(γ-D-Glutamyl)-*meso*-2(L),2′(D)-diaminopimelic Acid as the Minimal Prerequisite Structure of FK-156: Its Acyl Derivatives with Potent Immunostimulating Activity

Sir:

FK-156 (1) is a recently discovered immunostimulating



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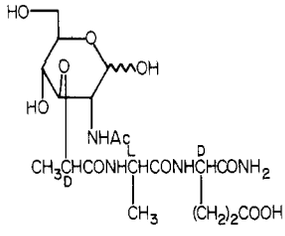

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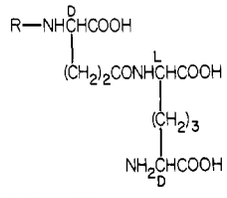
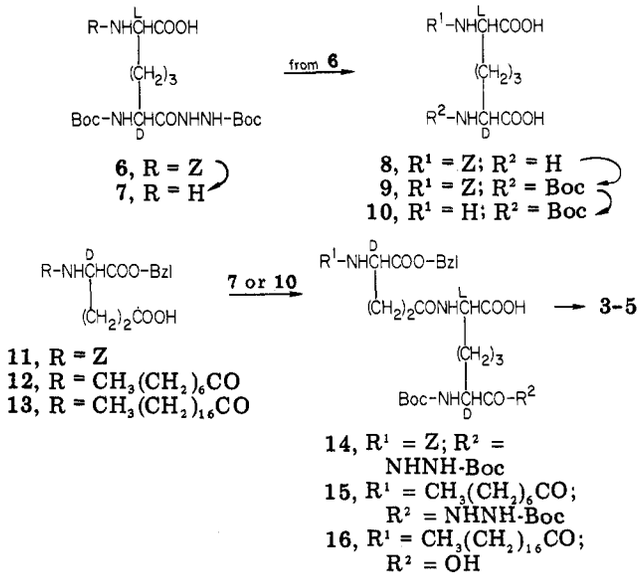


3, R = H
4, R = $CH_3(CH_2)_6CO$
5, R = $CH_3(CH_2)_{16}CO$

microbial metabolite with a structurally close resemblance to the bacterial cell-wall peptidoglycan peptides.[1] The structure of 1 is unique, as compared with the well-known muramyl dipeptide (MDP, 2), in the respect that 1 is devoid of the glucosamine residue at the *O* terminal of the D-lactoyl side chain and instead carries the *meso*-2,2′-diaminopimelylglycine residue at the γ-C terminal of the D-glutamic acid in 1. Of particular interest is its wide range of immunological activities despite the lack of the muramyl moiety which had, until recently, been considered to be essential for the biological activity.[2,3] Therefore, it was considered that the 2,2′-diaminopimelic acid in 1, like the muramyl moiety in 2, might be a major contributor to the unique immunoactivity. As part of a program on this natural product, we were interested in preparing the minimal prerequisite structure that might include this important component, 2,2′-diaminopimelic acid. We also hoped to obtain smaller molecular active fragments that would be more practical in preparation on a sufficiently large scale. Herein we report the preparation of N^2-(γ-D-glutamyl)-*meso*-2(L),2′(D)-diaminopimelic acid (3) as the minimal structural unit capable of eliciting the biological response characteristic of 1.[4] Its caprylyl derivative 4 proved to be a potential broad-spectrum immunostimulant as efficient as 1; also, the stearoyl derivative 5 showed a potent tumor-suppressive activity that was not present in the original compound 1.

Scheme I

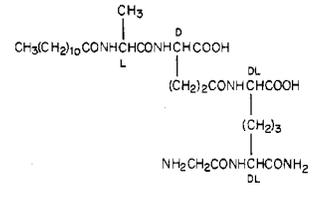


The compounds of interest were prepared as outlined in Scheme I. α-Benzyl carbobenzyloxy-D-glutamate (11)[5]

(1) (a) Isolation: T. Gotoh, Y. Kuroda, M. Okumura, T. Tanaka, T. Nishiura, M. Kohsaka, H. Aoki, and H. Imanaka, Interscience Conference on Antimicrobial Agents and Chemotherapy, 21st, Chicago, IL, p 414, 1981, abstr. (b) Synthesis: K. Hemmi, H. Takeno, S. Okada, O. Nakaguchi, Y. Kitaura, and M. Hashimoto, *J. Am. Chem. Soc.*, **103**, 7026 (1981). (c) Activity: Y. Watanabe, Y. Mine, Y. Yokota, S. Tawara, M. Hashimoto, M. Nishida, S. Goto, and S. Kuwahara, in ref 1a, p 413.

(2) See, for example, E. Lederer, *J. Med. Chem.*, **23**, 819 (1980).

(3) Recently, Migliore-Samour et al. have also reported an immunostimulating activity of lauroyl tetrapeptide i: D. Migliore-Samour, J. Bauchaudon, F. Floc'h, A. Zerial, L. Ninet, G. H. Werner, and P. Jollès, *C. R. Hebd. Seances Acad. Sci., Ser. D*, **289**, 473 (1979); *Life Sci.*, **26**, 889 (1980).



(4) The partial structures, *N*-[*N*-(D-lactoyl)-L-alanyl]-D-glutamic acid and *N*-[2(L),2′(D)-diaminopimel-1-yl]glycine, were found to be substantially inactive in any bioassays described here.

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Table I. Influence on Carbon Clearance in DDY Mice (Male)[a]

compd	dose, mg/kg	K (mean ± SE)	stimulation index (K_t/K_c)
controls		0.020 ± 0.003	1.0
1	0.1	0.029 ± 0.001	1.5
	1	0.038 ± 0.007*	1.9
3	1	0.021 ± 0.005	1.1
	10	0.058 ± 0.010**	2.9
4	0.1	0.032 ± 0.009	1.6
	1	0.048 ± 0.009**	2.4
5	0.1	0.032 ± 0.006	1.6
	1	0.048 ± 0.005**	2.4

[a] Clearance from the blood was measured according to the method described by Biozzi et al.[15] Compounds were administered to mice (five animals in each series) sc 24 h before injecting a colloidal carbon suspension (170 mg/mL) at a dose of 1 mL/100 g by the same route (* = $p < 0.05$ and ** = $p < 0.01$ as compared to their controls).

Table II. Induction of Delayed-Type Hypersensitivity to Egg Albumin in Hartley Guinea Pigs (Male)[a]

compd	dose, μg/site	skin response (mean ± SE)
controls		0
1	1	8.2 ± 1.7**
	10	10.0 ± 1.3**
3	1	3.3 ± 1.4*
	10	6.9 ± 2.0**
4	1	8.3 ± 1.5**
	10	3.7 ± 1.6
5	1	1.7 ± 1.1
	10	3.1 ± 1.3*

[a] Guinea pigs (five animals in each series) were immunized in both hind footpads with 1 mg of egg albumin with compounds in Freund's incomplete adjuvant (FIA). After 2 weeks, 5 μg of egg albumin was given as challenge, and skin reactions (diameter of induration, mm) were measured after 48 h (* = $p < 0.05$ and ** = $p < 0.01$ as compared to their controls).

Table III. Suppression Effect[a] of Meth-A Fibrosarcoma in BALB/c Mice (Female)[b]

compd	dose, μg/site	suppression[c]
controls		0/10
1	100	0/10
5	100	9/9

[a] Reference 16. [b] A mixture of Meth-A (1×10^5 cells) and compounds dissolved (1) or suspended (5)[17] in a 0.5% solution of methylcellulose in saline was inoculated intradermally into mice. Results were obtained on the 28th day after the tumor inoculation. [c] Number of tumor-free mice/number of mice tested.

was preactivated with isobutyl chloroformate (*N*-methylmorpholine/CH_2Cl_2, −10 to −15 °C) and coupled, at −10 to 0 °C, to the silyl ester of 7,[6] prepared by treatment with bis(trimethylsilyl)acetamide in CH_2Cl_2–DMF (room temperature), giving the condensation product **14** as a foam: $[\alpha]_D$ +14.5° (*c* 0.4, AcOH); 89% yield. Removal

Table IV. Protective Effect against *Escherichia coli* Infection in ICR Mice (Male)[a]

compd	dose, mg/kg	survival[b]
controls		1/10
1	0.1	6/10
	1	7/10
3	0.1	4/10
	1	4/10
4	0.1	7/10
	1	6/10
5	0.1	7/10
	1	8/10

[a] Compounds were administered to mice ip on the 4th day before challenging *E. coli* 22 (7.2×10^7) by the same route. Results were obtained on the 3rd day after the bacterial challenge. [b] Number of survivors/number of mice tested.

of the protecting groups in **14** by alkaline hydrolysis (1 N NaOH/aqueous MeOH, room temperature),[7a,8] treatment with trifluoroacetic acid (room temperature),[7b] oxidation with $NaIO_4$ (2.5 equiv, H_2O, pH 1 with 0.1 N H_2SO_4, 0 °C),[7c] and hydrogenolysis (10% Pd/C, H_2O)[7d] yielded compound **3** as a powder: $[\alpha]_D$ −17.0° (*c* 0.2, H_2O); TLC R_f 0.13 (A), 0.64 (B);[10] 52% yield.[11] Compound **4** was prepared from α-benzyl caprylyl-D-glutamate **12**[12] (mp 79–90 °C) using a similar procedure: coupling **12** to **7** gave **15** [mp 145–147 °C; $[\alpha]_D$ −17.5° (*c* 0.2, $CHCl_3$); 86% yield], which was deprotected by hydrogenolysis,[7e] treated with trifluoroacetic acid,[7b] and oxidized with HIO_4[7c] to provide **4** [mp 182–184 °C dec; $[\alpha]_D$ −9.7° (*c* 0.2, H_2O); TLC R_f 0.37 (A), 0.34 (C);[10] yield 78%].[13]

For preparing compound **5**, the same route described above was found to be inapplicable because of the sparing solubilities of the intermediates in the deprotection processes. This problem was, however, solved by removal of the hydrazide protecting group of *meso*-2,2′-diaminopimelic acid in an early step before introduction of the fatty acid containing fragment. Thus, **6**,[6] which is the precursor of **7**, was treated with trifluoroacetic acid[7b] and oxidized with HIO_4[7c] in a manner similar to the one described above to give **8** [mp ~245 °C dec; $[\alpha]_D$ −12.7° (*c* 0.2, AcOH); TLC R_f 0.40 (A);[10] yield 76%]. Reprotection of **8** with the Boc group (Boc-ON/Et_3N/acetone–H_2O) to give **9** [foam; quantitative yield] and subsequent hydrogenolysis[7d] of **9** in AcOH gave **10** [mp >250 °C; $[\alpha]_D$ +21.5° (*c* 0.2, AcOH); TLC R_f 0.38 (A);[10] yield 81%]. Then, α-benzyl stearoyl-D-glutamate (**13**)[12] [mp 80–81 °C] was

(5) G. H. L. Nefkens and R. J. F. Nivard, *Recl. Trav. Chim. Pays-Bas*, **83**, 199 (1953).
(6) The preparations of **6** and **7** have been described in our preceding paper.[1b]
(7) Treatments for removal of (a) Bzl, (b) Boc, (c) hydrazide, (d) Z, and (e) Z and Bzl, respectively.
(8) It was conceivable that alkaline hydrolysis of the γ-glutamyl peptides would coproduce the α-rearrangement byproducts as in the case of the rearrangement of glutamine methyl ester to isoglutamine.[9] In the preparation of **3**, however, this kind of byproduct, even if coproduced, most probably was removed by two Dia-ion HP-20 column chromatographies after oxidation with HIO_4 and hydrogenolysis, respectively.
(9) E. Sondheimer and R. W. Holley, *J. Am. Chem. Soc.*, **76**, 2467 (1954).
(10) Analytical thin-layer chromatography was performed with silica gel 60 F_{254} (E. Merck AG) using the following solvent systems: A, *n*-BuOH–AcOH–H_2O (5:2:3); B, *n*-PrOH–H_2O (3:2); C, *n*-PrOH–H_2O (7:3).
(11) Anal. Calcd for $C_{12}H_{21}N_3O_7 \cdot 1.5H_2O$: C, 41.60; H, 6.98; N, 12.13. Found: C, 41.20; H, 6.84; N, 12.27. Amino acid analysis: Glu, 1.00; A_2pm, 1.03.
(12) Prepared from α-benzyl D-glutamate by acylation with caprylyl chloride or stearoyl chloride.
(13) Anal. Calcd for $C_{20}H_{35}N_3O_8 \cdot 0.5H_2O$: C, 52.85; H, 7.98; N, 9.25. Found: C., 52.65; H, 7.88; N, 9.25. Amino acid analysis: Glu, 1.00; A_2pm, 1.11.

similarly coupled to **10**, giving **16** [mp 125–127 °C; $[\alpha]_D$ −3.7° (*c* 0.2, $CHCl_3$); yield 89%], which was finally deprotected by hydrogenolysis[7a] in AcOH and trifluoroacetic acid treatment[7b] to produce **5** [mp 194–198 °C dec; $[\alpha]_D$ −7.9° (*c* 0.4, AcOH); TLC R_f 0.39 (A), 0.40 (C),[10] yield 89%].[14]

In Table I is given the phagocytic activity in DDY mice in the carbon clearance assay. An increase in the rate of carbon clearance was observed in mice treated with 1 mg/kg of **1**. In contrast, **3** showed an increase at a dose of 10 mg, thus retaining the activity, though less than **1**. Compounds **4** and **5**, on the other hand, showed activities of the same order as or rather superior to **1** at 1 mg/kg. The enhancing effect on the potency by introduction of the fatty acid residues to **3** is noteworthy.

As shown in Table II, all the three new compounds also displayed some effects in induction of delayed-type hypersensitivity to egg albumin in Hartley guinea pigs. As compared with **1**, **3** was somewhat less potent, while **4** showed the same order of activity at a dose of 1 μg/site. Compound **5** did not so markedly affect the adjuvant potency in this assay system. This last case might deserve more experimental investigation, because **5**, on the other hand, exhibited a potent tumor-suppressive property as can be seen in Table III. In fact, when Meth-A fibrosarcoma in BALB/c mice was used, **5** was shown to be highly effective in suppressing the tumor growth, while compound **1** was entirely inactive. There might possibly be no parallel relationship between the adjuvanticity and the tumor-suppression activity.

Table IV shows the results of an experiment on antiinfectious effect in ICR mice against *Escherichia coli* 22. Compound **3** was moderately active but again slightly less than **1**. Noticeable is that **4** and **5** were both potent comparably to **1**, thus serving satisfactorily as substitutes for **1** in stimulating the antibacterial resistance.

In summary, except for the tumor suppression test, compound **3** represents the minimal active structure essential for eliciting the effects in the immunostimulating assays so far examined. Its derivatives **4** and **5** were found to be capable of increasing resistance to bacterial infection as efficiently as **1**, and **5** proved to possess the unique tumor-suppression ability lacking in **1**. On the basis of these activity profiles, **4** and **5** are now undergoing more detailed examinations for antiinfectious and antitumor effectiveness, respectively.

(14) Anal. Calcd for $C_{30}H_{55}N_3O_8 \cdot 1.5H_2O$: C, 58.80; H, 9.54; N, 6.86. Found: C, 58.72; H, 9.70; N, 6.68. Amino acid analysis: Glu, 1.00; A_2pm, 1.09.

(15) G. Biozzi, B. Benacerraf, and B. N. Halpern, *Br. J. Exp. Pathol.*, **34**, 441 (1953).

(16) In a regression test, **5** also showed a significant effect on regression of the tumor on systemic administration to mice already bearing the tumor. This will be reported in due course.

(17) For convenience in preparation of the suspension, **5** was converted into its HCl salt [mp ~125 °C dec; $[\alpha]_D$ −9.0° (*c* 0.2, AcOH). Anal. Calcd for $C_{30}H_{55}N_3O_8 \cdot HCl \cdot H_2O$: C, 56.27; H, 9.13; N, 6.56; Cl, 5.54. Found: C, 55.88; H, 9.07; N, 6.61; Cl, 5.17].

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2-Arylpyrazolo[4,3-*c*]quinolin-3-ones: Novel Agonist, Partial Agonist, and Antagonist of Benzodiazepines

Sir:

We wish to report on three novel 2-arylpyrazolo[4,3-*c*]quinolin-3-ones, all of which possess extremely high affinity for the benzodiazepine receptors, yet the first (**3a**) is a very potent antagonist of diazepam, the second (**3b**) is a safe antianxiety agent, and the third (**3c**) is a hitherto unknown partial agonist of the benzodiazepine receptor.

In 1977, Squires and Braestrup[1a] and Möhler and Okada[1b] reported on the high-affinity binding sites for benzodiazepines in rat brain tissues. Since that time, the receptors have been found in several mammalian species, including humans.[1b,2] The purpose of the existence of such receptors is not clearly understood, but obviously their function is not merely to receive benzodiazepine derivatives. Rather, they happen to show high affinity for various benzodiazepine derivatives, such as chlordiazepoxide and diazepam, as well as for still unknown endogenous ligands with unknown biological importance.[3] While search for the yet elusive endogenous ligands continues,[4] a number of synthetic compounds with diverse structures have been found to possess high affinity for the benzodiazepine receptors,[5] and some of them were reported to be antagonistic toward the physiological effects of benzodiazepine anxiolytics.[6] It is important to learn the physiological properties and structural requirements of various benzodiazepine–receptor binders (agonists, antagonists, and

(1) (a) R. F. Squires and C. Braestrip, *Nature (London)*, **266**, 732 (1977); (b) H. Möhler and T. Okada, *Science*, **198**, 849 (1977).

(2) (a) Braestrup, R. Albrechtsen, and R. F. Squires, *Nature (London)*, **269**, 702 (1977); (b) C. Braestrup and R. F. Squires, *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 3805 (1977); (c) C. Braestrup and R. F. Squires, *Eur. J. Pharmacol.*, **48**, 263 (1978).

(3) There have been a number of reports on putative endogenous ligands for the benzodiazepine receptors. However, they do not rigorously meet the criteria of endogenous ligands. For reviews, see (a) P. J. Marangos, S. M. Paul, F. K. Goodwin, and P. Skolnick, *Life Sci.*, **25**, 1093 (1979), and (b) I. L. Martin, *Trends Neurosci.*, **3**, 299 (1980).

(4) (a) L. G. Davis, H. McIntosh, and D. Reker, *Pharmacol. Biochem. Behav.*, **14**, 839 (1981); (b) J. H. Woolf and J. C. Nixon, *Biochemistry*, **20**, 4243 (1981).

(5) (a) A. S. Lippa, D. Critchett, M. C. Sano, C. A. Klepner, E. N. Greenblatt, J. Coupet, and B. Beer, *Pharmacol. Biochem. Behav.*, **10**, 831 (1979); (b) J. C. Blanchard, A. Boireau, C. Garret, and L. Julou, *Life Sci.*, **24**, 2417 (1978); (c) C. Braestrup, M. Nielsen, and C. E. Olsen, *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 2288 (1980); (d) Guérémy, and A. Uzan, *Life Sci.*, **28**, 1439 (1981).

(6) (a) S. S. Tenen and J. D. Hirsch, *Nature (London)*, **288**, 609 (1980); (b) P. J. Cowen, A. R. Green, D. J. Nutt, and I. L. Martin, *ibid.*, **290**, 54 (1981); (c) P. Skolnick, S. Paul, J. Crawley, K. Rice, S. Barker, R. Weber, M. Cain, and J. Cook, *Eur. J. Pharmacol.*, **69**, 525 (1981). (d) J. N. Crawley, P. J. Marangos, S. M. Paul, P. Skolnick, and F. K. Goodwin, *Science*, **211**, 725 (1981). (e) W. Hunkeler, H. Möhler, L. Pieri, P. Polc. E. P. Bonetti, R. Cumin, R. Schaffner, and W. Haefely, *Nature (London)*, **290**, 514 (1981); (f) A. J. Czernick, B. Petrack, C. Tsai, F. R. Granat, R. K. Rinehart, H. J. Kalinsky, R. A. Lovell, and W. D. Cash, "High-Affintiy Occupancy of Benzodiazepine Receptors by the Novel Antagonist CGS 8216", ASPET/PSC Meeting, Calgary, Canada, 1981; *Pharmacologist*, **23**, 160 (1981); (g) P. Bernard, K. Bergen, R. Sobiski, and R. D. Robson, "CGS 8216—An Orally Effective Benzodiazepine Antagonist", ASPET/PSC Meeting, Calgary, Canada, 1981; *Pharmacologist*, **23**, 150 (1981); (h) A. Darragh, M. Scully, R. Lambe, I. Brick, C. O'Boyle, and W. W. Downie, *Lancet*, **2**, 8 (1981); (i) H. Möhler, W. P. Burkard, H. H. Keller, J. G. Richards, and W. Haefely, *J. Neurochem.*, **317**, 714 (1981).

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