Sepharose CL-6B column were pooled and used as a source of purified CANP inhibitor.

The molecular weight of this inhibitor was estimated to be $3.5\text{–}4.0 \times 10^5$ by gel filtration on Sepharose CL-6B according to a previously described procedure[12]. The inhibitor for CANP was heat stable since after heating at 98 °C for 20 min, more than 80% of its inhibitory activity remained. The inhibitory effect is apparently not due to chelation of Ca^{++} since the inhibition was not reversed by a high concentration of Ca^{++} (10 mM). Furthermore, the activity of trypsin, α-chymotrypsin, plasmin, thrombin, papain or ficin was not suppressed by the inhibitor, indicating its specificity to platelet CANPs.

As shown in figure 2, activities of μ-CANP and m-CANP were decreased in proportion to the amount of CANP inhibitor, which indicated that the inhibitor interacted stoichiometrically with CANP in a manner similar to a formation of the α_2-macroglobulin-protease complex[17]. In order to see whether inhibitor and CANPs form a high molecular weight complex in the absence of Ca^{++}, platelet extract was applied to the Sephadex G-200 column equilibrated with buffer containing EGTA. Inhibitor was eluted separately from CANPs, thus indicating a requirement for Ca^{++} in inhibitor-CANP complex formation (results not shown). When inhibitor was first preincubated with CANPs in the absence of Ca^{++} and then tested in the standard assay system containing 0.4 mM $CaCl_2$ or 4.0 mM $CaCl_2$, a similar decrease in activities of μ-CANP and m-CANP was observed. Thus, preincubation in the absence of Ca^{++} did not affect inhibitory activity.

Discussion. In the present study, a high molecular weight endogenous inhibitor of platelet CANPs was identified and characterized. This inhibitor was found to be specific for platelet CANPs in that it did not interfere with other enzymes, such as trypsin, etc. The interaction of the inhibitor with CANPs from other sources has not been studied. The newly described platelet CANP inhibitor is different from thiol protease inhibitors identified in the skin and leukocytes, since molecular weights of these inhibitors are low and they suppress the activity of papain or ficin[18,19].

Although CANP and its inhibitor were dissociated in the absence of Ca^{++} during gel chromatography on Sephadex G-200, the inhibitory effect was exerted by forming a stoichiometric complex of CANP and the inhibitor in the presence of Ca^{++}, indicating that Ca^{++} is involved in the binding of inhibitor to CANPs.

A similar inhibitor of m-CANP has been identified in cardiac muscle, brain and liver[14–16]. In these tissues, inhibition of μ-CANP by its endogenous inhibitor has not been studied but we have demonstrated in this study that the inhibitor can also block the activity of μ-CANP which is considered to be physiologically more significant than m-CANP because of its higher affinity for Ca^{++}.

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Synthesis and RES-stimulating activity of bacterial cell-wall peptidoglycan peptides related to FK-156

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Summary. The bacterial cell-wall peptidoglycan peptides and related compounds coupled with some fatty acids were synthesized, and their immunostimulating property was examined by carbon clearance assay. All the new compounds (**2–6**) proved to possess significant potencies superior to that of FK-156 (**1**).

A unique immunostimulating activity displayed by bacterial cell-wall peptidoglycans has attracted considerable attention in recent years[3]. In the preceding papers[4], we reported the synthesis of FK-156 (**1**), an immunostimulating microbial metabolite with a structurally close resemblance to the bacterial cell-wall peptidoglycan peptides. In connection with a major ongoing program on this immunostimulant, we were interested in preparing some analogues bearing D-analine instead of glycine in **1**, because such a peptide sequence comprises the fundamental unit of the peptidoglycan peptides in most gram-negative bacterial cell-walls[5]. Herein we report the syntheses of compounds (**2–6**) of this D-alanine series and their biological activity. The immunostimulating property of these compounds proved to be significantly superior to that of **1**.

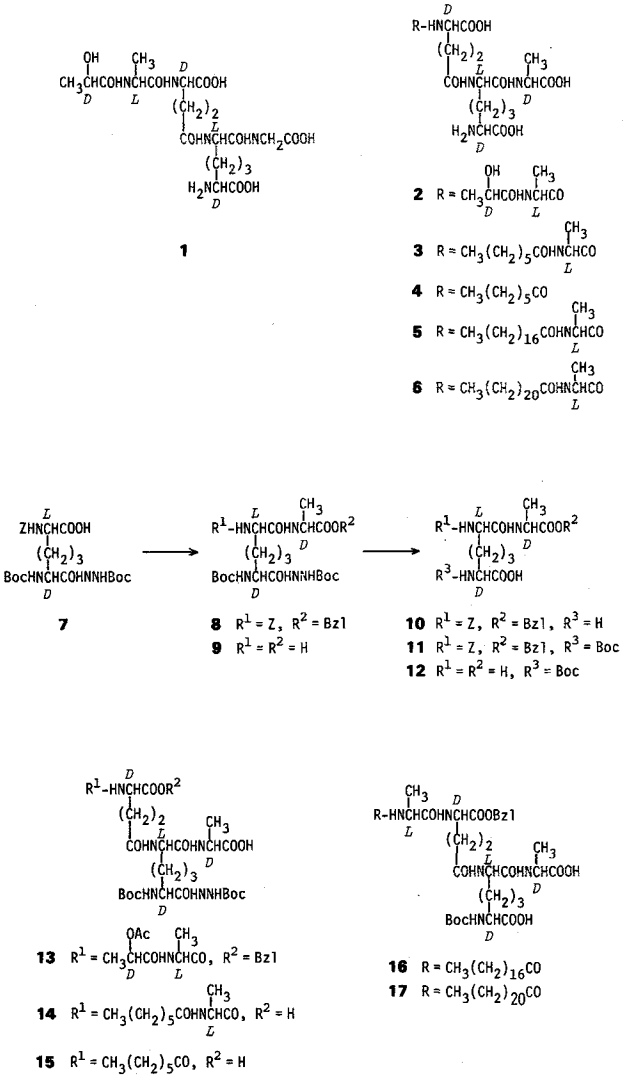
The compounds of interest were synthesized starting from

the intermediate **7** used for the synthesis of **1**[4a]. Thus, **7** was coupled to D-AlaOBzl via the mixed anhydride method (*i*-BuOCOCl) and the product **8** [powder, 80%] was then subjected to hydrogenolysis (10% Pd-C) to yield **9** [m.p. 142–145 °C (dec.), $[\alpha]_D + 43.7°$ (c 0.3, MeOH), R_f 0.68 (A)[6], 100%]. For the preparation of **2**, *O*-acetyl-D-lactoyl-L-Ala-D-Glu(OH)OBzl[4a] was coupled using *i*-BuOCOCl to the trimethylsilyl ester of **9**, prepared in situ by treatment with BSA, giving **13** (powder, 86%), which was deprotected by hydrogenolysis (10% Pd-C), alkaline hydrolysis (1N NaOH), treatment with TFA and oxidation with $NaIO_4$ (H_2O, pH 1) to afford **2** [powder, $[\alpha]_D -17.4°$(c 0.3, H_2O), R_f 0.59 (B), 56%]. Compound **3** was prepared from Z-L-Ala-D-Glu(OH)OBzl, which was coupled to the trimethylsilyl ester of **9** in a similar manner and the product was, after hydrogenolysis (10% Pd-C), acylated with heptanoic anhydride to yield **14** [m.p. ~90 °C (dec.), 83%]. Final deprotection (1. TFA; 2. $NaIO_4$) provided **3** [powder, $[\alpha]_D$ −26.0°(c 0.2, H_2O), R_f 0.39 (A), 62%]. Similarly, **4** [powder, $[\alpha]_D -15.7°$(c 0.5, MeOH), R_f 0.38, 82%] was prepared from Z-D-Glu(OH)OBzl via **15** (powder, 38%).

The compounds **5** and **6** were prepared by a somewhat modified route because of the inapplicability of the methods described above due to the low solubilities of intermediates in the oxidative deprotection process. Treatment of **8** with TFA, followed by oxidation with $NaIO_4$, gave **10** [m.p. ~215 °C (dec.), 79%], which in turn was reprotected with the Boc group by using BOC-ON and the product **11** [m.p. ~143 °C (dec.), 100%] was subjected to hydrogenolysis (10% Pd-C) to yield **12** [m.p. ~210 °C (dec.), $[\alpha]_D +27.1°$(c 0.4, AcOH), R_f 0.57 (A), 84%]. Coupling of stearoyl-L-Ala-D-Glu(OH)OBzl[7] to **12** by *N*-hydroxysuccinimide ester procedure gave **16** (m.p. 131–132 °C, 86%), which was deprotected (1. H_2/10% Pd-C; 2. HCl/AcOH) to provide **5** · HCl [powder, $[\alpha]_D -24.5°$ (c 0.2, AcOH), R_f 0.44 (A), 97%]. A similar sequence from behenoyl-L-Ala-D-Glu(OH)-OBzl[7] and **12** via **17** (m.p. 134–138 °C, 92%) provided **6** · HCl [m.p. ~180 °C (dec.), $[\alpha]_D -19.3°$(c 0.2, AcOH), R_f 0.42 100%].

The new compounds were tested by carbon clearance assay and their phagocytic effects are shown in comparison with that of **1** in the table. The reference compound **1** showed an increase in the rate of carbon clearance at a dose of 1 mg but not at a 0.1 mg dose. In contrast, all the new compounds showed significant stimulations even at 0.1 mg doses, thus proving to be at least 10 times more potent than **1**. The enhancing effect on the potency by replacement of glycine in **1** with D-alanine is noteworthy, although at this time no quantitative correlations can be drawn about structure-activity relationships among the compounds of this series. As can be seen from the data in the table, it is also obvious that the fatty acids serve satisfactorily as substitutes for the D-lactic acid residue in **1** and that the *N*-terminal L-alanine is not essential for the biological activity. Not listed in the table but found to retain the activity was N^2-(γ-D-glutamyl)-*meso*-2(L),2′(D)-diaminopimelic acid, whose fatty acid derivatives displayed activities of the same order as **1**[9], also demonstrating the needlessness of the L-alanine moiety and the effectiveness of the fatty acid residue for the biological activity.

All the compounds described above have been shown (though results are not given in detail here) to display excellent protective effects against bacterial infections and, especially the higher fatty acid derivatives **5** and **6**, to exhibit potent tumor-suppressive activities. These data will be reported elsewhere.



Influence on carbon clearance in DDY mice (male)*

Compound	Dose (mg/kg)	K-value (mean ± SE)	Stimulation index (Kt/Kc)
	0	0.021 ± 0.003	1.0
1	0.1	0.025 ± 0.005	1.2
	1	0.048 ± 0.004	2.3
	0	0.021 ± 0.003	1.0
2	0.1	0.054 ± 0.006	2.6
	1	0.059 ± 0.012	2.8
	0	0.025 ± 0.001	1.0
3	0.1	0.070 ± 0.002	2.8
	1	0.064 ± 0.001	2.6
	0	0.028 ± 0.002	1.0
4	0.1	0.073 ± 0.012	2.6
	1	0.072 ± 0.009	2.6
	0	0.011 ± 0.003	1.0
5	0.1	0.037 ± 0.005	3.4
	1	0.038 ± 0.007	3.5
	0	0.017 ± 0.005	1.0
6	0.1	0.053 ± 0.013	3.1
	1	0.052 ± 0.013	3.1

*Clearance of carbon from the blood was measured according to the method described by Biozzi et al.[8]. Compounds were administered to mice (5 animals in each series) via the s.c. route at 24 h before injecting a colloidal carbon suspension (170 mg/ml) with a dose of 1 mg/100 g by the same route.

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7 Prepared by stearoylation or behenoylation of L-Ala-D-Glu(OH)OBzl: stearoyl-L-Ala-D-Glu(OH)OBAl, m.p. 114-6 °C, $[\alpha]_D$ −26.7°(c 0.2, $CHCl_3$); behenoyl-L-Ala-D-Glu(OH)OBzl, m.p. 119-123 °C, $[\alpha]_D$ −14.1°(c 0.4, AcOH).
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Monoclonal antibodies against functionally distinct sites on the delta-endotoxin of *Bacillus thuringiensis* var. *thuringiensis*

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Summary. Monoclonal antibodies against the toxic units of *Bacillus thuringiensis* delta-endotoxin were raised by the hybridoma technique and detected by an indirect enzyme-linked immunosorbent assay (ELISA). Out of 5 positive clones, 1 was found to secrete antibodies which inactivate the toxin.

B. thuringiensis produces a proteinaceous crystal responsible for the insecticidal action of these bacteria towards many larvae belonging to the order Lepidoptera[2]. The crystal, also called protoxin, consists of dimeric subunits of mol.wt 230,000 and can be dissolved under alkaline or reducing conditions[3]. For biological activity a proteolytic activation is required, which occurs under natural conditions in the gut juice of susceptible insect larvae. In vitro digestion with trypsin rapidly leads to a fraction with mol.wt 80,000. It is degraded further to a more stable fragment with mol.wt 60,000 by prolonged exposure to protease. Both units are equally active on a molar basis. Low molecular weight by-products show no toxicity[4]. Little is known about the active site and the molecular mode of action of this toxin. Monoclonal antibodies are considered as a tool to unravel where the toxin acts and which sequences and/or conformation of the polypeptide chain are necessary for its action.

Materials and methods. B. thuringiensis var. *thuringiensis* (serotype H-1, strain LBG B4412) parasporal bodies were purified[5] and dissolved in 0.1 N NaOH for 30 min at 37 °C. The protoxin was digested for 3.5 h with sepharose-bound trypsin in 0.05 M carbonate buffer pH 9.5. The proteolytic fragments were separated on a Sephadex G-200 column using the same buffer. The toxic units, mol.wt 80,000 and 60,000, were eluted in 1 peak, concentrated and used as antigen.

3 female BALB/c mice were immunized twice. First 185 µg active delta-endotoxin in 0.25 ml complete Freund's adjuvant (CFA) were injected s.c., followed 6 weeks later by an i.p. boost with 90 µg of the toxic units in 0.1 ml 0.01 M phosphate buffered saline (PBS) pH 7.4.

A 2nd series of 3 female BALB/c mice was treated 4 times at weekly intervals. For the 1st injection 95 µg of delta-endotoxin in 0.125 ml CFA were given s.c., 2 more injections of 95 µg in 0.125 ml incomplete Freund's adjuvant (iCFA) followed also s.c. The boost of 90 µg in 0.1 ml PBS was given i.p.

4 days after boosting 2 separate fusions were done with spleen cells of mice of both immunization series (47S and 48S). $1.7.10^8$ primed spleen cells were fused with 3.10^7 SP2/O-Ag14 cells[6] with 50% polyethylenglycol[7] according to Köhler and Milstein[8]. Immediately after each fusion cells were diluted in 50 ml HAT medium[9], to which $5 \cdot 10^5$ peritoneal cells were added, and distributed in 96 wells of Costar 3524 trays.

Supernatants from growing hybrids were screened for anti-delta-endotoxin antibodies by the indirect method for microplate ELISA[10]. 200 ng antigen in 0.2 ml coating buffer[10] were incubated overnight in Cooke's Microtiter Plates (Dynatech Microelisa) at 4 °C. In a 2nd step 0.2 ml supernatant in different dilutions were added to the coated plates. Normal mouse serum and culture medium were taken as negative controls. Antibodies were allowed to react with the antigen for 2 h at 37 °C. Alkaline phosphatase was chosen as enzyme label and was coupled to goat antimouse IgG[10]. This conjugate, diluted 1:500, was incubated with the antigen-antibody complex for 2 h at 37 °C. P-nitrophenyl-phosphate (Sigma) was used as substrate. The rate of substrate degradation was examined by a Titertek Multiskan spectrophotometer at 405 nm after 30 min.

Positive cultures were cloned by limiting dilution according to Herbst and Braun[11]. Supernatants from growing clones were screened for anti-toxin antibodies and the positive clones were grown in DFCS culture medium[12]. Immunoglobulin subclasses were determined by immuno-diffusion of rabbit antiserum to the different mouse classes and subclasses (Bionetics) and 20 times concentrated supernatants of positive clones[11]. SDS-PAGE with ^{14}C-leucine labeled supernatants[13] was carried out according to

Feeding inhibition of *Pieris brassicae* larvae in percentage as a function of toxin concentration and antibody containing supernatants from different clones

Supernatant applied	Toxin (ng/larvae) 0	5	10	20	40
Culture medium	0	100	100	100	100
47S94.2	0	0	0	10	40
48S34.1	0	100	100	100	100
48S44.1	0	100	100	100	100
48S54.1	0	80	100	100	100
48S54.4	0	100	100	100	100