8

Low-Molecular-Weight Immunomodifiers Produced by Micro-organisms

HAMAO UMEZAWA

Institute of Microbial Chemistry, 3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

Introduction: initiation of the study

It has often been said that micro-organisms produce compounds which suppress the growth of other micro-organisms; a great many antibiotics have thus been discovered. However, this assertion has not yet been proved experimentally: moreover, the production of an antibiotic depends on fermentation conditions. It is possible that many genes which cause the production of microbial products of various chemical structures have been generated, with subsequent production of these secondary metabolites which are not necessary for the growth of micro-organisms. Quantitatively exact screening methods make it highly probable that the target compound can be detected in culture filtrates.

By 1965, NMR and X-ray crystallography had been introduced into natural-product chemistry, and it became possible to elucidate quickly the structures of microbial products. Moreover, at about that time, there was a rapid increase in our understanding of the biochemistry of various diseases. We therefore initiated the screening of culture filtrates for low-molecular-weight (LMW) enzyme inhibitors. The first report was issued in 1969, since when more than 50 inhibitors have been discovered in this laboratory (Umezawa, 1982); their structures have been determined, and structure–activity relationships and biosyntheses have been studied. The isolation of so many inhibitors of various enzymes is an indication of the multiplicity of compounds, produced by micro-organisms, with different structures and pharmacological activities.

Abbreviations: ADCC, antibody-dependent cellular toxicity; A_2pm, diaminopimelyl; BCG, Bacille Calmette-Guérin; CFU, colony-forming unit; Con A, concanavalin A; CSF, colony-stimulating factor; DTH, delayed-type hypersensitivity; IF, interferon; IL, interleukin; IMC, Institute of Microbial Chemistry; i.v., intravenous; LMW, low molecular weight; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; NK, natural killer; PHA, phytohaemagglutinin; PWM, pokeweed mitogen; SRBC, sheep red blood cells; TNF, tumour-necrosis factor.

Biotechnology and Genetic Engineering Reviews — Vol. 3, September 1985
0264–8725/85/03/255–19$10.00 + $0.00 © Intercept Ltd

In a study of the influence of known antibiotics on immune responses it was found that coriolin and diketocoriolin B (which were discovered in this laboratory) increased the number of mouse spleen cells producing antibody to sheep red blood cells (SRBC). In another study, coriolin and diketocoriolin B were shown to inhibit Na^+–K^+-ATPase (EC 3.6.1.37). These observations indicated that compounds which bind to surfaces or membranes of cells might modify immune responses: screening was therefore carried out for inhibitors of enzymes located on cell surfaces or membranes. Many enzymes, including aminopeptidases, alkaline phosphatases (EC 3.1.3.1.), and arylesterase (EC 3.1.1.2) were found to be located on surfaces of intact mammalian cells, but were not released extracellularly. In the course of this screening study, inhibitors of such enzymes were discovered and, as expected, most of them enhanced delayed-type hypersensitivity (DTH) to SRBC or increased the number of antibody-forming cells. Of the microbial products thus discovered, bestatin has been studied in most detail, and has been shown in clinical trials to be an effective adjunct to chemotherapy in prolonging the life span of cancer patients. With the exception of FK-156, the LMW immunomodifiers which are described in this article were discovered in our laboratory, and the data up to 1981 have been published in book form (Umezawa, 1981).

Immunity-enhancing compounds are potentially useful in the treatment of cancer and of infections in immune-deficient patients, while compounds suppressing immune reactions are of interest in the treatment of autoimmune diseases or in transplantation surgery.

Immunomodifiers produced by micro-organisms, and their actions

CORIOLIN AND DIKETOCORIOLIN B

Coriolins A and C (*Figure 1*) are antineoplastic antibiotics produced by the fungus *Coriolus consors* (Takeuchi *et al.*, 1969). Diketocoriolin B (*Figure 1*), a derivative of coriolin B, is also produced by *C. consors* (Takahashi *et al.*, 1971; Takeuchi *et al.*, 1971). Coriolins A and C and diketocoriolin B inhibit the growth of tumour cells *in vitro* and prolong the survival period of mice bearing L-1210 and other murine experimental tumours. Investigation of their cytotoxic action has shown that they inhibit Na^+–K^+-ATPase, a membrane enzyme (Kunimoto, Hori and Umezawa, 1973).

Injection of 0·1–0·01 μg/mouse* of diketocoriolin B at the time of immunization was found to increase the number of mouse spleen cells producing antibody to SRBC (Ishizuka *et al.*, 1972). Although coriolins A and C also increase the number of antibody-forming cells, the effect of diketocoriolin B on antibody formation has been studied in most detail. The effect of diketocoriolin B in increasing the numbers of antibody-forming cells was also

* Throughout this chapter, mean weight of mice used = 20 g and mean volume of cultures = 1 ml.

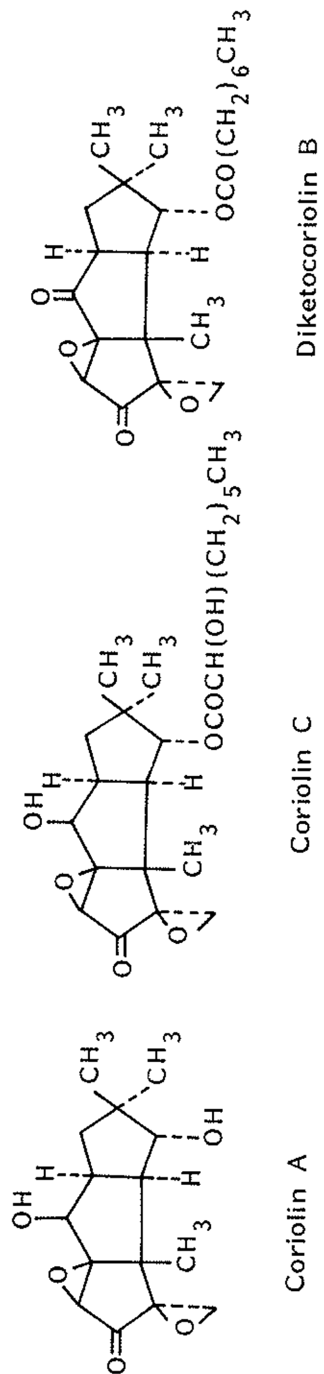

Figure 1. Coriolins A, C and diketocoriolin B.

observed in spleen-cell cultures (Ishizuka, Takeuchi and Umezawa, 1981): the addition of 0·01–1 ng/culture to spleen-cell cultures increased the number of antibody-forming cells. The greatest stimulus to antibody formation was observed when diketocoriolin B was added 1 or 2 days after the start of the culture. The removal of adherent cells (macrophages) or treatment with anti-T-cell serum and complement did not alter the effect of diketocoriolin B. Moreover, antibody formation by spleen cells of athymic mice was also augmented by addition of diketocoriolin B (0·1–1 ng/culture). Thus it has been shown that diketocoriolin B directly acts on B cells, stimulating their proliferation or their differentiation into antibody-producing cells.

The antineoplastic effect of diketocoriolin B on L-1210 was observed at doses of 1–100 μg/mouse i.p.: the optimal dose for enhancing antibody formation was much lower than that for inhibiting tumour growth. As described above, diketocoriolin B inhibits Na^{+}–K^{+}-ATPase: this reaction on the cell membrane may be the stimulus which induces antibody formation in cells able to do so; the author therefore initiated an investigation into those microbial products which bind to cell membranes.

BESTATIN

In order to find microbial products which bind to cells involved in immune responses, we decided to search for inhibitors of enzymes located on cell surfaces. As already mentioned, in this study, aminopeptidases (Aoyagi *et al.*, 1976), alkaline phosphatases and arylesterase were found to be located on cell surfaces but not released extracellularly.

A search for inhibitors of aminopeptidase B (arginine aminopeptidase; EC 3.4.11.6) was carried out as follows. The reaction mixture was prepared by mixing 0·25 ml of 2 mM L-arginine-β-naphthylamide (Protein Research Foundation, Japan), 0·5 ml of 0·1 M Tris-HCl buffer at pH 7·0 and 0·1 ml distilled water with or without inhibitor, in a series of test tubes in a 37°C bath. After 3 minutes, 0·15 ml of aminopeptidase B solution was added and mixed well. Exactly 30 minutes later, 1·0 ml of a solution of the stabilized diazonium salt Garnet GBC (1 mg/ml) in 1 M acetic acid buffer at pH 4.2, containing 10% Tween 20, was added. After the mixture had stood for 15 minutes at room temperature, absorbancy was read at 525 nm. The amount of the enzyme was adjusted to give an optical density of around 0·35, that is, about 25 nmol of β-naphthylamine was released. The reaction was also carried out without addition of enzyme solution and the result was taken as blank. The concentration of the inhibitor required for 50% inhibition (IC_{50}) was calculated.

This procedure led to the discovery of an inhibitor, which was named bestatin (Umezawa *et al.*, 1976a). The strain producing bestatin was classified as *Streptomyces olivoreticuli*. The structure of bestatin (*Figure 2*) was determined as (2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutyryl)-L-leucine (Suda *et al.*, 1976b; Nakamura *et al.*, 1976) and the compound was chemically synthesized (Suda *et al.*, 1976c). Bestatin inhibits aminopeptidase B ($K_i = 6{\cdot}0 \times 10^{-8}$ M) and leucine aminopeptidase (amino-peptidase (cytosol); EC

Bestatin

Amastatin

Forphenicine

Esterastin

Ebelactone A: R=CH_3

Ebelactone B: R=CH_3CH_2

Figure 2. Bestatin, amastatin, forphenicine, esterastin and ebelactones.

3.4.11.1; $K_i = 2{\cdot}0 \times 10^{-8}$M). As expected, bestatin augmented immune responses and exhibited an antineoplastic effect against some experimental tumours (Umezawa *et al.*, 1976b). Bestatin and all its stereoisomers were synthesized: isomers which had the 2*S*-configuration in the 3-amino-2-hydroxy-4-phenylbutyryl moiety were found to have DTH-enhancing activity equivalent to that of bestatin, whereas 2*R*-stereoisomers had little, if any, inhibitory effect on aminopeptidase B and showed only a slight enhancement of DTH (Suda *et al.*, 1976a). The immunity-enhancing activity of bestatin was not dose-dependent, and the degree of activity of the stereoisomers in enhancing DTH paralleled their activity in inhibiting aminopeptidases. Thus, the action of bestatin in enhancing immune responses was suggested to be due to its binding to cells involved in immunity. Binding to mouse lymphocytes has been confirmed, using ^{3}H-bestatin. After 10 minutes' exposure, 4×10^6 molecules of bestatin bound to one lymphocyte at 37°C, and 3×10^5 at 0°C. Müller *et al.* (1982) have confirmed that the number of bestatin molecules binding to macrophages ($1459 \pm 95 \times 10^4$/macrophage) was markedly greater than that binding to T cells ($245 \pm 17 \times 10^4$/T cell), whereas the number binding to B cells was much smaller ($92 \pm 6 \times 10^4$/B cell) than that to T cells. Bestatin at 1–1000 μg/mouse enhanced DTH to oxazolone.

As reported by Müller *et al.* (1982) intraperitoneal injection of bestatin (5 or 50 mg/kg) increased the activity of DNA-directed DNA polymerase α in T cells, but not in B cells; DNA-directed DNA polymerase β activity, however, was not increased. Terminal deoxynucleotidyl transferase in bone marrow was also stimulated (Müller *et al.*, 1979; Umezawa, 1981; all three enzymes are EC 2.7.7.7).

The administration of bestatin at the time of immunization with SRBC or other antigens augmented DTH over a wide dose range. At a high dosage level (50 mg/kg) it increased the number of antibody-forming cells in a spleen-cell population. In immunosuppressed mice to which cyclophosphamide had been given, or in which Ehrlich ascites tumour or Sarcoma 180 had been implanted, bestatin restored the DTH response (Ishizuka *et al.*, 1980a). Treatment with bestatin augmented humoral and cellular immune responses in aged mice (Bruley-Rosset *et al.*, 1979).

Bestatin has a mitogenic action to lymphocytes both *in vivo* and *in vitro* (Ishizuka *et al.*, 1980b). Proliferation of splenic lymphocytes is stimulated by oral administration of bestatin. The administration of bestatin increased the incorporation of ^{3}H-thymidine into lymphoid organs such as thymus, spleen and lymph nodes, but not into other organs. Increased incorporation was observed in T cells of spleen and thymus but not in B cells (Umezawa, 1981). In cultures of whole spleen cells a low level of bestatin (0·1–1 μg/ml) increased the incorporation of ^{3}H-thymidine into lymphocytes (Ishizuka *et al.*, 1980b), but this effect disappeared when macrophages were depleted or T cells were destroyed, indicating that at low levels bestatin stimulates proliferation of T cells in the presence of macrophages. A high level of bestatin (more than 100 μg/ml) stimulated lymphocyte proliferation. In this case, depletion of T cells did not affect the bestatin effect, indicating that a high level of bestatin may stimulate B cells through activation of macrophages.

The addition of bestatin to macrophage cultures stimulated interleukin 1 (IL 1) production. Interleukin 2 (IL 2) production in lymphocyte cultures induced by concanavalin A (Con A) was also enhanced by bestatin. Bestatin stimulates IL 2 production by human peripheral blood mononuclear cells and increases the IL 2 sensitivity of IL 2-dependent cultured T cells (Noma *et al.*, 1984).

Bestatin also enhanced the production of colony-stimulating factor (CSF) to generate macrophage-granulocytes (CFU) in bone-marrow-progenitor cell cultures. Bestatin enhances production of CFU in bone marrow cell cultures supplemented with CSF (Ishizuka *et al.*, 1980a). The administration of bestatin to mice in which leucopenia had been induced by cytotoxic agents such as mitomycin C or cyclophosphamide prevented the occurrence of leucopenia and prolonged the survival period.

Bestatin inhibited the growth of murine transplantable tumours through activation of host defence mechanisms (Ishizuka *et al.*, 1980a; Umezawa, 1981; Abe *et al.*, 1984). Oral administration of bestatin on days 8 to 12 after tumour inoculation was much more effective than treatment immediately after transplantation for the control of IMC carcinomas and C1498 myeloid leukaemias. Moreover, this antineoplastic effect was observed at doses of 0·05–5 mg/kg but not at over 50 mg/kg.

The antineoplastic effect of bestatin against the tumours described above was markedly reduced in athymic mice. Splenic lymphocytes from bestatin-treated tumour-bearing mice inhibited the corresponding tumour growth. As bestatin has no cytotoxic effect at 100 μg/ml, this antitumour effect of bestatin can be attributable only to the activation of a host defence system.

Antibody-dependent cellular toxicity (ADCC) and natural killer (NK) cell activities of spleen cells of tumour-bearing mice were enhanced by bestatin administration (Abe *et al.*, 1984). NK activity in human peripheral blood was enhanced by bestatin both *in vitro* and *in vivo* (Umezawa, 1981). Macrophages taken from normal and tumour-bearing mice given bestatin have cytostatic activity against the growth of tumour cells *in vitro*. Macrophage activation by bestatin, which resulted in the inhibition of lymphomas and P815 mastocytoma cells, has also been reported (Schorlemmer, Bosslet and Sedlacek, 1983).

Bestatin administration (twice a week for 15 weeks from the start of carcinogen treatment) retarded tumour growth in the generation of skin cancer by 20-methylcolanthrene and prolonged the survival period significantly (Ishizuka *et al.*, 1980a). The effect of bestatin on MNNG (*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine)-induced stomach cancer in rats has also been examined. With various injection schedules (e.g. twice a week over a period of 1 to 85 weeks) bestatin inhibited stomach cancer development at 85 weeks after carcinogen treatment.

As described above, bestatin augments cell-mediated immunity over a wide dose range, and antibody formation at higher doses. Bestatin stimulates T-cell proliferation in spleen cells *in vivo* through the activation of macrophages. It induces IL 1 production by peritoneal macrophages and augments IL 2 production by Con A- or phytohaemagglutinin- (PHA)-stimulated

spleen cells. Production of cytokines (colony-stimulating factors and macrophage-stimulating factor) is induced by bestatin. Bestatin exhibits an antineoplastic effect against syngeneic murine tumours through the activation of a host defence system. Bestatin has no cytotoxicity *in vitro* and has extremely low toxicity *in vivo*. Antineoplastic effectors such as macrophages, NK cells and T cells are activated or induced by the administration of bestatin.

The results of the clinical study of bestatin may be summarized as follows:

1. Daily doses of 30 or 60 mg increased the percentage and number of T cells which had been reduced in cancer patients;
2. Activities of NK and helper T cells were restored and the numbers of mitomycin C-sensitive suppressor cells in cancer patients were reduced by bestatin;
3. Suppressor activity in serum of cancer patients against pokeweed mitogen- (PWM)-induced IgG formation by human peripheral blood lymphocytes was prevented by bestatin;
4. The number of nucleated cells, which had been reduced in the bone marrow of cancer patients, was increased;
5. Negative skin reactions against various antigens in patients who had lost the ability to respond to antigens such as tuberculin, turned to positive.

The therapeutic effects of bestatin have been confirmed by randomized tests on leukaemia, melanoma, tumours of the head and neck, oesophagus, bladder, etc. For instance, in patients over 40 years of age, with myeloic leukaemia, marked suppression of relapses and prolongation of the survival period has been confirmed. Bestatin also effectively suppresses infections.

AMASTATIN

Tests of the ability of *Streptomyces* culture filtrates to inhibit human serum aspartate aminopeptidase A (EC 3.4.11.7), led to the discovery of an inhibitor which was named amastatin (Aoyagi *et al.*, 1978a), and which inhibited aspartate aminopeptidase A ($K_i = 15 \times 10^{-8}$M) and leucine aminopeptidase (cytosol aminopeptidase; EC 3.4.11.1) ($K_i = 160 \times 10^{-8}$M). The structure was determined (*Figure 2*) and amastatin was chemically synthesized (Tobe *et al.*, 1979). The (2*S*,3*R*)-3-amino-2-hydroxypropionyl moiety is present in amastatin as well as in bestatin. All stereoisomers of amastatin at the asymmetric centres of the (2*S*,3*R*)-3-amino-2-hydroxy-5-methylhexanoyl moiety have been synthesized and their 50% inhibition concentrations against aminopeptidase A have been determined (Tobe *et al.*, 1982): for the 2*S*-3*R* isomer (amastatin) this was 1·1 μg/ml; for the 2*S*-3*S* isomer, 10 μg/ml; for the 2*R*–3*S* isomer, 53 μg/ml, and for the 2*R*-3*R* isomer, 180 μg/ml. These results indicate that the *S*-configuration at the 2-*C* of the 3-amino-2-hydroxy-5-methylhexanoyl moiety of amastatin has an important role in the inhibition of aminopeptidase A.

Intraperitoneal injection of 10–100 μg/mouse of amastatin increased the number of spleen cells producing antibody to SRBC.

ESTERASTIN AND EBELACTONES A AND B

In studies of the enzyme activities of intact cells, not only aminopeptidases but also arylesterase (EC 3.1.1.2) and alkaline phosphatases (EC 3.1.3.1) were found to be located on cell surfaces; these enzymes were not released extracellularly. Screening tests on culture filtrates led to the discovery of an inhibitor of hog pancreas esterase (Umezawa *et al.*, 1978). This inhibitor, named esterastin, was shown to have the structure presented in *Figure 2* (Kondo *et al.*, 1978). The strain producing esterastin was classified as *Streptomyces lavendulae*. Esterastin inhibited arylesterase very strongly: the K_i against arylesterase was $1{\cdot}6 \times 10^{-10}$M (*p*-nitrophenyl acetate was used as substrate); it suppressed DTH and decreased the number of mouse spleen cells producing antibody to SRBC.

In culture filtrates of another strain (*Streptomyces aburaviensis*) two other arylesterase inhibitors were discovered and named ebelactones A and B ($K_i = 9{\cdot}2 \times 10^{-8}$M and 5×10^{-10}M, respectively; Umezawa *et al.*, 1980). Their structures were found to be as shown in *Figure 2* (Uotani *et al.*, 1982a). Uotani *et al.* (1982b), using ^{13}C, have shown that A is biosynthesized from one molecule of acetic acid and six of propionic acid; B from one molecule of acetic acid, five of propionic acid and one of butyric acid. Ebelactones inhibited not only esterase but also formylmethionine aminopeptidase; 50% inhibition concentrations were 0·08 μg/ml for A and 0·02 μg for B. Ebelactone A enhanced DTH, but B did not. It is interesting that esterastin, which has a very low K_i value, suppresses immune responses whereas ebelactone B, which also has a very low K_i value, does not suppress or enhance immune responses.

FORPHENICINE

In screening for inhibitors of chicken intestine alkaline phosphatase, forphenicine was discovered (Aoyagi *et al.*, 1978b). This was produced by *Streptomyces fluvoviridis* var. *acarbodicus*, and was found to have the structure shown in *Figure 2*, i.e. (4-formyl-3-hydroxyphenyl)-glycine (Yamamoto *et al.*, 1978); it has since been synthesized (Morishima *et al.*, 1982). It inhibits chicken intestine alkaline phosphatase very strongly ($K_i = 1{\cdot}6 \times 10^{-7}$M) but this inhibition is not substrate competitive, the type of inhibition being uncompetitive.

Forphenicine exhibits an antineoplastic effect through activation of the host defence system (Umezawa, 1981). The intraperitoneal injection of forphenicine at the time of immunization enhances DTH over a wide dose range (1–1000 μg/mouse). In the case of immunosuppressed mice treated with cyclophosphamide or inoculated with Ehrlich ascites tumour, injection of forphenicine prevented the reduction of DTH responses. Forphenicine

increased the number of anti-SRBC antibody-forming cells in mouse spleen when it was given at the time of immunization. Antibody formation by spleen-cell cultures was also augmented by addition of forphenicine at dose levels of 0·1–1 μg/ml. The effect of forphenicine in enhancing antibody formation by cultured spleen cells has been examined in forphenicine-pretreated macrophage-rich populations with or without the addition of lymphocyte-rich cell populations and, conversely, in forphenicine-pretreated lymphocyte-rich cell populations with or without the addition of macrophage-rich cell populations. The enhancing effect was observed only in cultures composed of the pretreated macrophage-rich cell populations and lymphocytes. This indicates that forphenicine stimulates macrophages and enhances antibody formation.

Forphenicine stimulated the proliferation of splenic T lymphocytes in the presence of macrophages (Umezawa, 1981). Whole spleen cells were incubated with forphenicine in a medium containing fetal calf serum for 24 hours; lymphocytes collected by sedimentation were then cultured for 72 h in the presence of ^{3}H-thymidine: ^{3}H-thymidine incorporation was increased about threefold by this treatment. This effect of forphenicine was markedly reduced in spleen-cell populations lacking adherent cells or in those treated with anti-T-cell serum and complement.

Forphenicine also enhances phagocytosis of yeast by peritoneal macrophages and granulocytes.

Forphenicine exhibits antineoplastic action against murine syngeneic tumours. When mice were inoculated subcutaneously with 10^6 IMC carcinoma cells into the groin, the subsequent forphenicine treatment exhibited a stronger inhibitory effect against tumour growth when started 8 days after tumour-cell inoculation than when the treatment was started one day after tumour-cell inoculation; the effect was observed at doses of 0·05–5 mg/kg. As forphenicine has low toxicity for mice (LD_{50} = >500 mg/kg) and no cytotoxicity at 100 μg/ml, the antineoplastic effect is host mediated through forphenicine-activated defence systems.

FORPHENICINOL

The forphenicine derivative forphenicinol (L-(3-hydroxy-4-hydroxymethylphenyl)glycine) contains a hydroxymethyl group instead of the formyl group of forphenicine (Morishima *et al.*, 1982). Forphenicinol inhibits alkaline phosphatases very weakly, but it binds to cells and, unlike forphenicine, enhances immune responses when given orally (Ishizuka *et al.*, 1982a).

Oral administration of forphenicinol given at the time of immunization augmented DTH to SRBC or oxazolone. Forphenicinol restored the impaired immune responses in mice to which cyclophosphamide had been given or into which a tumour had been inoculated. Forphenicinol differed from bestatin and forphenicine in that the treatment of whole spleen cell populations with forphenicinol did not stimulate proliferation of lymphocytes. The oral administration of forphenicinol stimulated phagocytosis of yeast by thioglycollate-elicited peritoneal macrophages (Ishizuka *et al.*, 1982a).

The administration of forphenicinol before eliciting interferon (IF) by a second injection of BCG augmented production of IF compared with BCG alone. After priming with BCG, the injection of cyclophosphamide markedly reduced IF production; the administration of forphenicinol prevented this reduction and significantly restored IF production (H. Umezawa, unpublished work). Production of tumour-necrosis factor (TNF) in BCG-primed mice was also enhanced by administration of forphenicinol. Interleukin 1 (IL 1) production was augmented by administration of forphenicinol.

Forphenicinol has antineoplastic activity against experimental tumours (Ishizuka *et al.*, 1982b). The oral administration of 0·08–0·31 mg/kg daily for 10 days starting 5 days after the subcutaneous inoculation of Ehrlich carcinoma cells markedly inhibited tumour growth. Forphenicinol at doses of 0·05–5 mg/kg daily for 6 days starting 8 days after inoculation of solid tumours of the IMC carcinoma inhibited tumour growth by about 70%. Forphenicinol enhanced the antitumour effect of 6-mercaptopurine against Ehrlich carcinoma, of aclacinomycin against IMC carcinoma, and of cyclophosphamide against L-1210 and C3H/HeN mammary carcinomas. As forphenicinol has a low toxicity (LD_{50} = >500 mg/kg i.p. in mice) and no detectable cytotoxicity, its antitumour effect is attributable to the activation of host defence systems. Recent results have indicated that the administration of forphenicinol to normal mice activates macrophages so that they become cytostatic to tumour cells, while in tumour-bearing mice forphenicinol stimulates concomitant immunity against inoculated tumours and activates T cells and/or macrophages.

The administration of forphenicinol together with mitomycin C prevented the reduction of the number of leucocytes in peripheral blood that normally follows treatment with mitomycin C, and prolonged the survival period. Forphenicinol increased the number of CFU in bone marrow cell cultures in the presence of colony-stimulating factor. Forphenicinol is now undergoing clinical trials.

FK-156 AND ITS DERIVATIVES

FK-156, an immunostimulator which prevents bacterial infections, is produced by *Streptomyces olivaceogriseus* sp. nov. and *Streptomyces violaceus* (Gotoh *et al.*, 1982a,b; Kawai *et al.*, 1982). It is a water-soluble acidic tetrapeptide, D-Lac-L-Ala-γ-D-Glu-(L)-*meso*-α,ε-A_2*pm*(L)-GlyOH (*Figure 3*). LD_{50} of FK-156 was estimated to be more than 1 g/kg when administered intravenously to mice. This substance exhibits antitumour activity against animal tumours (Izumi *et al.*, 1983)

Intradermal injection of FK-156 with ovalbumin as antigen emulsified in Freund's incomplete adjuvant augmented DTH and humoral antibody formation in guinea pigs. After various doses (0·1–100 μg/site) had been tested, the optimal dose was suggested to be 1–10 μg/site for both immune responses. It also enhanced carbon clearance activity of phagocytic cells in peripheral blood of mice. The phagocytic index in mice was enhanced by 0·01–10 mg/kg

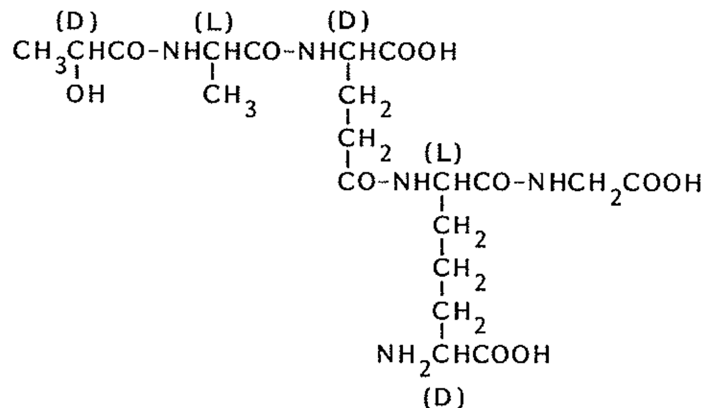

Figure 3. FK-156.

of FK-156 given one day before the assay, and the optimum dose was 1–10 mg/kg.

The effect of FK-565 (a derivative of FK-156) on phagocytes in normal and immunosuppressed mice has been studied (Mine *et al.*, 1983a,b). Subcutaneous injection 1 day before harvesting the cells increased the number of polymorphonuclear cells, while similar administration 4 or 7 days before harvest elevated the number of macrophages in the peritoneal cavity. Injection of FK-156 enhanced the chemotactic activity of polymorphonuclear cells about fivefold and macrophages about elevenfold. Polymorphonuclear cells (PMN) or macrophages from mice given FK-156 were incubated with *Pseudomonas aeruginosa*, *Listeria monocytogenes*, or *Candida albicans* and, after incubation, the number of viable micro-organisms in sonicates of the phagocytes was counted. Treatment with FK-156 was thus shown to enhance phagocytosis and the ability of these cells to kill micro-organisms. Treatment with FK-156 restored the number of phagocytes in the peritoneal cavity of mice dosed with cyclophosphamide or hydrocortisone. Chemotactic activity of polymorphonuclear cells in mice which had been given 50 mg of hydrocortisone daily for 4 days before the test was suppressed markedly but recovered on treatment with FK-156. Chemotaxis suppressed by the addition of the supernatant of sonicated S180 cells to macrophage cultures was also restored to normal levels by the addition of FK-156 at 10 μg/ml.

The addition of FK-156 to spleen-cell cultures increased the incorporation of ^{3}H-thymidine into cultured cells.

Antineoplastic activities of FK-156 against AH66 rat hepatomas and P388 mouse leukaemias have been reported (Gotoh *et al.*, 1982b; Izumi *et al.*, 1983). Intraperitoneal injection of FK-156 (0·1, 0·3 and 1·0 mg/kg/day for 5 days starting 9 days before tumour-cell inoculation) prolonged the survival period of rats with AH66 hepatoma cells implanted intraperitoneally. Injection of FK-156 or FK-565 into P388 solid tumours at days 6 and 9 after tumour inoculation inhibited tumour growth by about 50%, judging by the tumour

weight 14 days after tumour inoculation. Subcutaneous administration of FK-156 was less effective than injection into the tumour. As FK-156 and FK-565 had low cytotoxicity against P388 leukaemia cells in culture, the antineoplastic effect of these substances may be attributable to a host-mediated mechanism.

The administration of FK-156 and FK-565 to mice by parenteral or oral routes before challenge with micro-organisms showed a protective effect against *E. coli, L. monocytogenes, P. aeruginosa*, and others (Mine *et al.*, 1983b; Yokota *et al.*, 1983).

Effect of oxanosine, bestatin, forphenicinol and arphamenine on the suppressor system

In routine experiments testing for the effect of compounds in inhibiting tumour growth, daily administration of the test compound is started on the day after tumour inoculation and is continued for 7 or 10 days. As described in the previous section, additional treatment with bestatin or other immunity-enhancing agents enhances the effect of cytotoxic antineoplastic compounds and increases the number of surviving mice. Moreover, the survivors often gain immunity to this tumour and reject a second inoculation of the same type of tumour cells. However, to promote a similar synergistic effect between immunity-enhancing and cytotoxic substances in the treatment of human cancer, it may be necessary to inhibit the generation or action of suppressor cells, because these cells are increased in number and/or activity in cancer patients. For this reason, we have initiated a search for microbial products that inhibit the generation or action of suppressor cells.

The belief that certain cytotoxic antibiotics may inhibit the generation or growth of suppressor cells more strongly than that of other immune cells is supported by the observation that certain antineoplastic drugs such as cyclophosphamide, 6-mercaptopurine and aclacinomycin, have been reported to inhibit the generation or action of suppressor cells in mice. Oxanosine (*Figure 4*), developed in our laboratory, is a new type of nucleoside

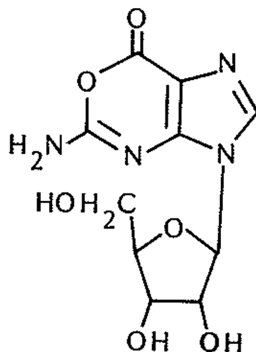

Figure 4. Oxanosine, a new antineoplastic antibiotic produced by *Streptomyces capreolus*.

antineoplastic antibiotic (Nakamura *et al.*, 1981; Shimada *et al.*, 1981; Yagisawa *et al.*, 1982; Yagisawa, Takita and Umezawa, 1983). Oxanosine has been shown to enhance both DTH and antibody formation, by inhibition of the generation of suppressor cells. The suppressor activity of transferred spleen cells was markedly reduced by oxanosine treatment. One intraperitoneal injection of oxanosine (50 mg/kg) 6 or 12 days after the subcutaneous inoculation of IMC-carcinoma cells (10^6) was found to inhibit tumour growth equally as well as cyclophosphamide. Oxanosine (0·06–4 mg/mouse) enhanced the phagocytic activity of peritoneal macrophages.

It should also be possible to find compounds inhibiting the production or action of suppressor cells among LMW immunomodifiers. As is well known, the intravenous injection of a large number (e.g. 10^8) of SRBC into mice induces the production of suppressor cells. The transfer of 5×10^7 mouse spleen cells from mice 5 days after the intravenous (i.v.) injection of 10^8 SRBC showed marked suppression of DTH as judged by the footpad test. The oral administration of bestatin (1, 10 or 100 μg/mouse) on the same day as the i.v. administration of SRBC inhibited the generation of suppressor cells; that is, the footpad of bestatin-treated mice was significantly more swollen than in the control without bestatin treatment. Bestatin at 1, 10, or 100 μg/mouse daily for 2 days starting 2 days after the i.v. injection of 10^8 SRBC also inhibited the generation of suppressor cells. Similarly, forphenicinol and arphamenine (which will be described later) inhibited suppressor-cell generation caused by injection of a large number of SRBC. Bestatin at 1,10, or 100 μg/mouse given once on the day of the transfer of suppressor cells or daily for 3 days after the transfer of suppressor cells restored the reduced immunity caused by the transfer of suppressor cells. This indicates that bestatin administration also inhibits the action of suppressor cells. As described above, bestatin inhibits both production and action of suppressors which impede DTH.

Recently we have discovered two specific inhibitors of aminopeptidase B (Umezawa *et al.*, 1983): these compounds, named arphamenines A and B, have the structures shown in *Figure 5* (Ohuchi *et al.*, 1983). These inhibitors were isolated from culture filtrates of a bacterial strain classified as *Chromobacterium violaceum*. Structurally, arphamenine A is a methylene analogue of L-arginyl-L-phenylalanine, and B is that of L-arginyl-L-tyrosine; that is, instead of the amide group of the dipeptide, arphamenines contain a methylene ketone group, which biosynthetic studies have shown to be derived from acetate and to link the decarboxylated arginine and deaminated phenylalanine moieties of the molecule (Ohuchi *et al.*, 1984a). This is a most interesting example of the biosynthetic ability of micro-organisms.

Arphamenines and their analogues have been synthesized (Ohuchi *et al.*, 1984b).

Arphamenines A and B inhibit aminopeptidase B very strongly. The 50% inhibition concentrations are 0·006 μg/ml for A ($K_i = 2{\cdot}5 \times 10^{-9}$M) and 0·002 μg/ml for B ($K_i = 8 \times 10^{-10}$M). They inhibit neither aminopeptidase A nor cytosol aminopeptidase. Arphamenine B, which binds to aminopeptidase B more strongly than does arphamenine A (as shown by their K_i values), did

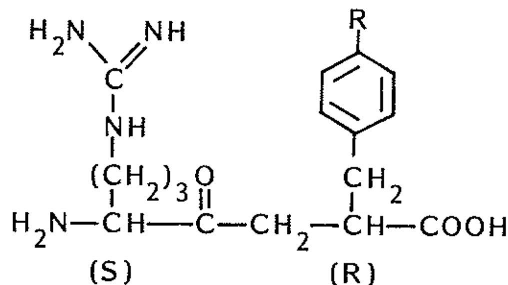

Figure 5. Arphamenines A (R=H) and B (R=OH).

not enhance DTH significnatly. Both arphamenine A and arphamenine B, at levels of 1·6 μg/ml or more, markedly inhibited the incorporation of ^{3}H-thymidine into mouse spleen cells stimulated by con A. Arphamenine A also inhibited the division of T blast cells produced by the addition of con A. In contrast, high concentrations (>6·25 μg/ml) of bestatin promoted the division of T blast cells. No effect on macrophage phagocytosis was observed for arphamenines: however, both arphamenines A and B showed significant inhibition against neoplasms such as IMC carcinoma, sarcoma 180 and Ehrlich carcinoma. As shown in *Table 1*, both arphamenines A and B inhibited the production of suppressor cells suppressing DTH: the effect of arphamenine A in enhancing DTH and the effects of arphamenines A and B

Table 1. Inhibition of suppressor-cell production by treatment with arphamenines A and B

Cells transferred	Treatment* (μg/mouse, i.p.)		DTH response** (× 0·1 mm)	Suppression (%)
Normal	None		8·0 ± 1·0	
Suppressors*	None		4·1 ± 0·4	49
Suppressors	Arphamenine A	10	6·4 ± 0·2	20
Suppressors		100	6·0 ± 0·3	25
Suppressors		1000	5·3 ± 0·4	34
Suppressors	Arphamenine B	10	5·7 ± 0·2	29
Suppressors		100	7·8 ± 0·5	2
Suppressors		1000	9·9 ± 0·4	0

* After suppressor cells were induced by intravenous injection of 10^{8} sheep red blood cells (SRBC), 4·5 × 10^{7} spleen cells containing suppressor cells were transferred to normal mice (mean weight 20 g) and the delayed-type hypersensitivity (DTH) reaction was elicited by injection of 10^{5} SRBC. Two days after the 10^{8} SRBC injection for suppressor induction, arphamenine was given intraperitoneally once a day for 3 days, and 4·5 × 10^{7} spleen cells were again transferred, to assess the DTH response.
** Increase in footpad thickness.

in inhibiting certain mouse tumours may be attributable to their effects on the suppressor system.

All the LMW immunity-enhancing compounds, such as bestatin, forphenicinol and arphamenine, had a strongly inhibitory effect against the IMC carcinoma when given 6 and 7 days after the subcutaneous inoculation of 10^6 tumour cells, but were almost inactive when given 3 days after inoculation of the tumour cells. The greater inhibition by these compounds when given at the later stage suggests that the antineoplastic effect could result from inhibition of the production or action of suppressor cells.

Conclusion

Compounds inhibiting the production or action of suppressor cells should enhance the effect of chemotherapy in eliminating neoplasms. If suppressor cells are thus inhibited, then LMW compounds which enhance the activities of NK-cells or macrophages should be better able to reinforce the effect of chemotherapy. The residual tumours which remain after macroscopic elimination could thus be eliminated by administration of immunity-enhancing agents which restore the reduced immunity in cancer patients, or by additional chemotherapy. Use of the proper combination of different immunity-enhancing agents such as bestatin, forphenicine, forphenicinol, arphamenines, etc. may produce a stronger therapeutic effect than when any one is used alone.

As shown by the results of the study of low-molecular-weight (LMW) enzyme inhibitors and LMW immunomodifiers, not only antibiotics but also other compounds which have various pharmacological activities can be found in cultured broths of micro-organisms. In order to obtain such compounds, previously we have been dependent on micro-organisms isolated from soils or other natural materials. Now, it may be said that the new age is coming, where new micro-organisms and new products will be prepared by the application of modern biotechnology.

References

ABE, F., SHIBUYA, K., UCHIDA, M., TAKAHASHI, K., HORINISHI, H., MATSUDA, A., ISHIZUKA, M., TAKEUCHI, T. AND UMEZAWA, H. (1984). Effect of bestatin on syngeneic tumors in mice. *Gann* **75**, 89–94.

AOYAGI, T., SUDA, H., NAGAI, M., OGAWA, K., SUZUKI, J., TAKEUCHI, T. AND UMEZAWA, H. (1976). Aminopeptidase activities on the surface of mammalian cells. *Biochimica et biophysica acta* **452**, 131–143.

AOYAGI, T., TOBE, H., KOJIMA, F., HAMADA, M., TAKEUCHI, T. AND UMEZAWA, H. (1978a). Amastatin, an inhibitor of aminopeptidase A, produced by actinomycetes. *Journal of Antibiotics* **31**, 636–638.

AOYAGI, T., YAMAMOTO, T., KOJIRI, K., KOJIMA, F., HAMADA, M., TAKEUCHI, T. AND UMEZAWA, H. (1978b). Forphenicine, an inhibitor of alkaline phosphatase produced by actinomycetes. *Journal of Antibiotics* **31**, 244–246.

BRULEY-ROSSET, M., FLORENTIN, I., KIGER, N., SCHULZ, J. AND MATHÉ, G. (1979).

Restoration of impaired immune functions of aged animals by chronic bestatin treatment. *Immunology* **38**, 75–83.

GOTOH, T., NAKAHARA, K., IWAMI, M., AOKI, H. AND IMANAKA, H. (1982a). Studies on a new immunoactive peptide, FK-156. I. Taxonomy of the producing strains. *Journal of Antibiotics* **35**, 1280–1285.

GOTOH, T., NAKAHARA, K., NISHIURA, T., HASHIMOTO, M., KINO, T., KURODA, Y., OKUHARA, M., KOHSAKA, M., AOKI, H. AND IMANAKA, H. (1982b). Studies on a new immunoactive peptide, FK-156. II. Fermentation, extraction and chemical and biological characterization. *Journal of Antibiotics* **35**, 1286–1292.

ISHIZUKA, M., TAKEUCHI, T. AND UMEZAWA, H. (1981). Studies on the mechanism of action of diketocoriolin B to enhance antibody formation. *Journal of Antibiotics* **34**, 95–102.

ISHIZUKA, M., IINUMA, H., TAKEUCHI, T. AND UMEZAWA, H. (1972). Effect of diketocoriolin B on antibody formation. *Journal of Antibiotics* **25**, 320–321.

ISHIZUKA, M., MASUDA, T., KANBAYASHI, N., FUKASAWA, S., TAKEUCHI, T., AOYAGI, T. AND UMEZAWA, H. (1980a). Effect of bestatin on mouse immune system and experimental murine tumors. *Journal of Antibiotics* **33**, 642–652.

ISHIZUKA, M., SATO, J., SUGIYAMA, Y., TAKEUCHI, T. AND UMEZAWA, H. (1980b). Mitogenic effect of bestatin on lymphocytes. *Journal of Antibiotics* **33**, 653–662.

ISHIZUKA, M., ISHIZEKI, S., MASUDA, T., MOMOSE, A., AOYAGI, T., TAKEUCHI, T. AND UMEZAWA, H. (1982a). Studies on effects of forphenicinol on immune responses. *Journal of Antibiotics* **35**, 1042–1048.

ISHIZUKA, M., MASUDA, T., KANBAYASHI, N., WATANABE, Y., MATSUZAKI, M., SAWAZAKI, Y., OHKURA, A., TAKEUCHI, T. AND UMEZAWA, H. (1982b). Antitumor effect of forphenicinol, a low molecular weight immunomodifier, on murine transplantable tumors and microbial infections. *Journal of Antibiotics* **35**, 1049–1054.

IZUMI, S., NAKAHARA, K., GOTOH, T., HASHIMOTO, S., KINO, T., OKUHARA, M., AOKI, H. AND IMANAKA, H. (1983). Antitumor effects of novel immunoactive peptides, FK-156 and its synthetic derivatives. *Journal of Antibiotics* **36**, 566–574.

KAWAI, Y., NAKAHARA, K., GOTOH, T., UCHIDA, I., TANAKA, H. AND IMANAKA, H. (1982). Studies on a new immunoactive peptide, FK-156. III. Structure elucidation. *Journal of Antibiotics* **35**, 1293–1299.

KONDO, S., UOTANI, M., MIYAMOTO, M., HAZATO, T., NAGANAWA, H., AOYAGI, T. AND UMEZAWA, H. (1978). The structure of esterastin, an inhibitor of esterase. *Journal of Antibiotics* **31**, 797–800.

KUNIMOTO, T., HORI, M. AND UMEZAWA, H. (1973). Mechanism of action of diketocoriolin B. *Biochimica et biophysica acta* **298**, 513–525.

MINE, Y., WATANABE, Y., TAWARA, S., YOKOTA, Y., NISHIDA, M., GOTO, S. AND KUWAHARA, S. (1983a). Immunoactive peptides, FK-156 and FK-565. III. Enhancement of host defense mechanisms against infection. *Journal of Antibiotics* **36**, 1059–1066.

MINE, Y., YOKOTA, Y., WAKAI, Y., FUKUDA, S., NISHIDA, M., GOTO, S. AND KUWAHARA, S. (1983b). Immunoactive peptides, FK-156 and FK-565. I. Enhancement of host resistance to microbial infection in mice. *Journal of Antibiotics* **36**, 1045–1050.

MORISHIMA, H., YOSHIZAWA, J., USHIJIMA, R., TAKEUCHI, T. AND UMEZAWA, H. (1982). Synthesis of forphenicinol and forphenicine. *Journal of Antibiotics* **35**, 1500–1506.

MÜLLER, W. E. G., ZAHN, R. K., ARENDES, J., MUNSCH, N. AND UMEZAWA, H. (1979). Activation of DNA metabolism in T-cells by bestatin. *Biochemical Pharmacology* **28**, 3131–3137.

MÜLLER, W. E. G., SCHUSTER, D. K., ZAHN, R. K., MAIDHOF, A., LEYHAUSEN, G., FOLKE, D., KOREN, R. AND UMEZAWA, H. (1982). Properties and specificity of binding sites for the immunomodulator bestatin on the surface of mammalian cells. *International Journal of Immunopharmacology* **4**, 393–400.

NAKAMURA, H., SUDA, H., TAKITA, T., AOYAGI, T., UMEZAWA, H. AND IITAKA, Y. (1976). X-ray structure determination of (2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoic acid, a new amino acid component of bestatin. *Journal of Antibiotics* **29**, 204–207.

NAKAMURA, H., YAGISAWA, N., SHIMADA, N., TAKITA, T., UMEZAWA, H. AND IITAKA, Y. (1981). The X-ray structure determination of oxanosine. *Journal of Antibiotics* **34**, 1219–1221.

NOMA, T., KLEIN, B., CUPPISOL, D., YATA, J. AND SERROU, B. (1984). Increased sensitivity of IL2-dependent cultured T cells and enhancement of *in vitro* IL2 production by human lymphocytes treated with bestatin. *International Journal of Immunopharmacology* **6**, 87–92.

OHUCHI, S., SUDA, H., NAGANAWA, H., TAKITA, T., AOYAGI, T., UMEZAWA, H., NAKAMURA, H. AND IITAKA, Y. (1983). The structure of arphamenine A and B. *Journal of Antibiotics* **36**, 1576–1580.

OHUCHI, S., OKUYAMA, A., NAGANAWA, H., AOYAGI, T. AND UMEZAWA, H. (1984a). Biosynthetic studies of arphamenines A and B. *Journal of Antibiotics* **37**, 518–521.

OHUCHI, S., SUDA, H., NAGANAWA, H., KAWAMURA, K., AOYAGI, T. AND UMEZAWA, H. (1984b). Structure-activity relationships among derivatives of arphamenines, inhibitors of aminopeptidase B. *Journal of Antibiotics* **37**, 1741–1743.

SCHORLEMMER, H. U., BOSSLET, K. AND SEDLACEK, H. H. (1983). Ability of the immunomodulating dipeptide bestatin to active cytotoxic mononuclear phagocytes. *Cancer Research* **43**, 4148–4153.

SHIMADA, N., YAGISAWA, N., NAGANAWA, H., TAKITA, T., HAMADA, M., TAKEUCHI, T. AND UMEZAWA, H. (1981). Oxanosine, a novel nucleoside from actinomycetes. *Journal of Antibiotics* **34**, 1216–1218.

SUDA, H., AOYAGI, T., TAKEUCHI, T. AND UMEZAWA, H. (1976a). Inhibition of aminopeptidase B and leucine aminopeptidases by bestatin and its stereoisomers. *Archives of Biochemistry and Biophysics* **177**, 196–200.

SUDA, H., TAKITA, T., AOYAGI, T. AND UMEZAWA, H. (1976b). The structure of bestatin. *Journal of Antibiotics* **29**, 100–101.

SUDA, H., TAKITA, T., AOYAGI, T. AND UMEZAWA, H. (1976c). The chemical synthesis of bestatin. *Journal of Antibiotics* **29**, 600–601.

TAKAHASHI, S., NAGANAWA, H., IINUMA, H., TAKITA, T., MAEDA, K. AND UMEZAWA, H. (1971). Revised structure and stereochemistry of coriolins. *Tetrahedron Letters*, No. 22, 1955–1958.

TAKEUCHI, T., IINUMA, H., IWANAGA, J., TAKAHASHI, S., TAKITA, T. AND UMEZAWA, H. (1969). Coriolin, a new basidiomycetes antibiotic. *Journal of Antibiotics* **22**, 215–217.

TAKEUCHI, T., TAKAHASHI, S., IINUMA, H. AND UMEZAWA, H. (1971). Diketocoriolin B, an active derivative of coriolin B produced by *Coriolus consors*. *Journal of Antibiotics* **24**, 631–635.

TOBE, H., MORISHIMA, H., NAGANAWA, H., TAKITA, T., AOYAGI, T. AND UMEZAWA, H. (1979). Structure and chemical synthesis of amastatin. *Agricultural and Biological Chemistry* **43**, 591–596.

TOBE, H., MORISHIMA, H., AOYAGI, T., UMEZAWA, H., ISHIKI, K., NAKAMURA, K., YOSHIOKA, T., SHIMAUCHI, Y. AND INUI, T. (1982). Synthesis and structure-activity relationships of amastatin analogues, inhibitors of aminopeptidase A. *Agricultural and Biological Chemistry* **46**, 1865–1872.

UMEZAWA, H. (ED.) (1981). *Small Molecular Immunomodifiers of Microbial Origin—Fundamental and Clinical Studies of Bestatin*. Japan Scientific Societies Press, Tokyo/Pergamon Press, New York.

UMEZAWA, H. (1982). Low molecular weight enzyme inhibitors of microbial origin. In *Annual Review of Microbiology*, volume 36, pp. 75–99. Annual Reviews Inc., Palo Alto, California.

UMEZAWA, H., AOYAGI, T., SUDA, H., HAMADA, M. AND TAKEUCHI, T. (1976a). Bestatin, an inhibitor of aminopeptidase B, produced by actinomycetes. *Journal of Antibiotics* **29**, 97–99.

UMEZAWA, H., ISHIZUKA, M., AOYAGI, T. AND TAKEUCHI, T. (1976b). Enhancement of delayed-type hypersensitivity by bestatin, an inhibitor of aminopeptidase B and leucine aminopeptidase. *Journal of Antibiotics* **29**, 857–859.

UMEZAWA, H., AOYAGI, T., HAZATO, T., UOTANI, K., KOJIMA, F., HAMADA, M. AND TAKEUCHI, T. (1978). Esterastin, an inhibitor of esterase, produced by Actinomycetes. *Journal of Antibiotics* **31**, 639–641.

UMEZAWA, H., AOYAGI, T., UOTANI, K., HAMADA, M., TAKEUCHI, T. AND TAKAHASHI, S. (1980). Ebelactone, an inhibitor of esterase produced by actinomycetes. *Journal of Antibiotics* **33**, 1594–1596.

UMEZAWA, H., AOYAGI, T., OHUCHI, S., OKUYAMA, A., SUDA, H., TAKITA, T., HAMADA, M. AND TAKEUCHI, T. (1983). Arphamenine A and B, new inhibitors of aminopeptidase B, produced by bacteria. *Journal of Antibiotics* **36**, 1572–1575.

UOTANI, K., NAGANAWA, H., AOYAGI, T. AND UMEZAWA, H. (1982a). Biosynthetic studies of ebelactone A and B by ^{13}C NMR spectrometry. *Journal of Antibiotics* **35**, 1670–1674.

UOTANI, K., NAGANAWA, H., KONDO, S., AOYAGI, T. AND UMEZAWA, H. (1982b). Structural studies on ebelactone A and B, esterase inhibitors produced by actinomycetes. *Journal of Antibiotics* **35**, 1495–1499.

YAGISAWA, N., SHIMADA, N., TAKITA, T., ISHIZUKA, M., TAKEUCHI, T. AND UMEZAWA, H. (1982). Mode of action of oxanosine, a novel nucleoside antibiotic. *Journal of Antibiotics* **35**, 755–759.

YAGISAWA, N., TAKITA, T. AND UMEZAWA, H. (1983). A facile total synthesis of oxanosine, a novel nucleoside antibiotic. *Tetrahedron Letters* **24**, 931–932.

YAMAMOTO, T., KOJIRI, K., MORISHIMA, H., NAGANAWA, H., AOYAGI, T. AND UMEZAWA, H. (1978). The structure of forphenicine. *Journal of Antibiotics* **31**, 483–484.

YOKOTA, Y., MINE, Y., WAKAI, Y., WATANABE, Y., NISHIDA, M., GOTO, S. AND KUWAHARA, S. (1983). Immunoactive peptides, FK-156 and FK-565. II. Restoration of host resistance to microbial infection in immunosuppressed mice. *Journal of Antibiotics* **36**, 1051–1058.