USE OF PERFLUORINATED COUNTER IONS FOR THE COMBINATION OF ION PAIR HPLC AND FIELD DESORPTION MASS SPECTROMETRY

APPLICATION TO THE EARLY CHARACTERIZATION OF AMINOGLYCOSIDE ANTIBIOTICS

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The use of perfluorinated carboxylic acids counter ions is described for easy combination of ion pair HPLC and FD-MS. This technique is applied to the analysis of aminoglycoside antibiotics and to the problem of their early characterization from culture broths. Reliable molecular weight informations can be obtained with this method from minute amounts of purified products (10 μg) allowing formal identification in most cases.

The aminoglycoside (AG) antibiotics are an important family of therapeutic agents because of their activity against Gram-negative bacteria not readily susceptible to other antibiotics[1]. Their widespread use, clinical and in animal feed, has led to an increasing number of resistant bacterial strains, whose resistance usually results from enzymic modification of the antibiotic. There have been also some concerns about the toxicity of existing aminoglycosides. Thus, part of the current research on this group of antibiotics has been directed toward the discovery of new members hopefully less toxic and less susceptible to enzymic inactivation.

New members have thus been sought by synthesis, hemisynthesis, mutasynthesis and also by the screening of new microorganisms.

The recent discovery of fortimicins[2], an extremely promising family of aminoglycosides, substantiates the interest in this latter approach. To render this approach more efficient however, novel strategies have to be designed to increase the success rate for the discovery of new compounds[3]. In particular, one has to avoid rediscovering known products. Therefore, these strategies should aim at gaining, as early as possible, precise informations on the chemical structure of the antibiotics studied.

Preliminary insights into the nature of antibiotics produced by microorganisms can often been drawn from the analysis of their antimicrobial spectra[4,5]. Although this analysis is extremely valuable, definitive characterization and identification have to rely on physical and spectroscopical data.

TLC and bioautography have been extensively used for this purpose and characterization of antibiotics can be achieved using a number of solvent systems[6]. However, the important number of already discovered antibiotics has made this approach difficult to practice.

Spectroscopic techniques such as UV or IR have also been routinely employed for characterization of antibiotics, but they have found little use in the case of closely related compounds, such as aminoglycosides, which lack characteristic absorptions.

Relatively good insight could, however, be obtained through the knowledge of molecular weight

(MW) of the compounds. Indeed, MW is a basic characteristic of organic products. It has been established for most described antibiotics and, in addition, can be handled conveniently through computerized data banks.

MW determination can be best obtained by mass spectrometry (MS) using gentle ionization techniques such as field desorption (FD) which give mainly molecular ions[7]. FD-MS, in conjunction with powerful separation methods such as HPLC, is specially effective and has already proved useful in the environmental, medical and pharmaceutical field[8~10].

Complex buffers have however prevented ion pair HPLC to be conveniently combined with FD-MS. In this paper, we show that the use of perfluorinated carboxylic acid as counter ions allows easy coupling between these two techniques. We have applied this combination to the problem of early characterization of AG from culture broths.

Results

Coupling of Ion Pair HPLC with FD-MS

Due to the polar and basic nature of aminoglycosides, ion pair reverse phase high performance liquid chromatography has proved to be the method of choice for the separation of these antibiotics without prior derivatization. Although there are already a number of HPLC methods described which result in adequate separation of aminoglycosides[11,12], they generally use complex buffers which are difficult to remove from the sample. Moreover, these buffers, even in trace amounts, are inhibitors of the desorption process in the FD mode.

In a previous paper[13], we reported the use of perfluorinated carboxylic acids for the ion pair HPLC of AG using heptafluorobutyric, pentafluoropropionic and trifluoroacetic acid counter ions. We have now found that the use of these volatile counter ions allows, after chromatography, easy recovery of ultra pure micro samples of antibiotics readily analyzable by FD-MS. Recovery, as estimated by antimicrobial assay, is about 90% after HPLC. Interestingly, even when left with the sample, these perfluorinated counter ions are not inhibitor of the desorption process.

The operating conditions and results obtained with commercial pure antibiotics are shown in Table 1.

As expected, no ion was observed for streptomycin due to the presence of two guanidine residues. Butirosin, on the other hand did not give rise to a molecular ion but to a fragment peak at 355 due to the loss of 4-amino-2-hydroxybutyric acid. Satisfactory FD mass spectra were however recorded for all the other AG studied, using μg amounts of injected and recovered material. Reliable MW information can thus be obtained on a micro scale for most AG through coupling of ion pair HPLC and FD-MS. For the amounts used, sample handling is obviously important. We have found that good results can be obtained on 1 μg samples, when sample manipulation is reduced to a minimum (no transfers, no pipeting).

We then applied this method to the characterization, from crude bacterial extracts, of AG produced by fermentation. In a first step, known AG from various groups produced by reference strains were studied. In a second step unknown AG produced by wild strains of actinomycetes were investigated.

Characterization of AG Produced by Fermentation

After incubation of the bacteria, the antibiotics produced were first extracted with Amberlite IRC-50. The crude extract was chromatographed on a short Amberlite CG-50 column. An aliquot

Table 1. FD-MS analysis of pure micro samples of commercial AG recovered from ion pair HPLC.

Antibiotic	Solvent (MeOH - H_2O)*	Capacity factor k′	Amount of injected antibiotic (μg)	FD-MS analysis (m/z) $(M+H)^+$	Published MW
Streptomycin	62: 38 (a)	0.3	50	—	581
Kanamycin A	62: 38 (a)	0.8	15	485	484
Lividomycin A	62: 38 (a)	1.4	50	762	761
Ribostamycin	57: 43 (a)	2.6	20	455	454
Fortimicin A	57: 43 (a)	3.2	50 25	406	405
〃 B	57: 43 (a)	4	25	349	348
Apramycin	63: 37 (a)	1.2	15	540	539
Butirosin A	62: 38 (a)	0.6	50	356 (M−199)	555
Neomycin B	0: 100 (b)	9.3	15	615	614
Gentamicin C_{1a}	0: 100 (b)	2.6	10	450	449
〃 C_2	0: 100 (b)	5.2	30 10	464	463
〃 C_1	0: 100 (b)	11.3	10	478	477
Sisomicin	0: 100 (b)	2.3	15	448	447
Verdamicin	0: 100 (b)	3.5	15	462	461

* Chromatographic conditions: Aqueous phase, (a) 0.05 M heptafluorobutyric acid (pH 2.1), (b) 0.1 M trifluoroacetic acid (pH 1.7).

Table 2. Characterization by ion pair HPLC/FD-MS of AG produced by reference strains.

AG producing strain	AG produced	Volume of culture (m)	Solvent (MeOH - H_2O)[a]	Weight of CG-50 active fraction (mg)	Factor k′ of active compound	Amount of recovered material (μg)[b]	FD-MS (m/z) $(M+H)^+$	Theoretical MW
Micromonospora echinospora subsp. *inyoensis* NRRL 3292[21]	Sisomicin, verdamicin	800	60: 40	10	2.1	30	448, 462	447, 461
Streptomyces kanamyceticus ATCC 21352[22]	Kanamycin	30	57: 43	3	2.3	10	485	484
Micromonospora olivoasterospora NRRL 8178[2]	Gentamicins (C_{1a}, C_2, C_1)	30	63: 37	2	2.4	<10	450, 464, 478	449, 463, 477
Micromonospora purpurea NRRL 2953[23]	Fortimicins (A, B)	500	57: 43	8	3.2 4.0	40 40	406 350	405 349
Streptomyces ribosidificus ATCC 21294[24]	Ribostamycin	50	57: 43	6	2.6	>100	455	454

[a] Aqueous phase 0.05 M heptafluorobutyric acid (pH 2.1).

[b] Corresponding to a 1 mg injection and estimated from the area under the peak.

of the active fraction was analyzed by ion pair HPLC. The different components separated were tested for antimicrobial activity against *Bacillus subtilis*. The fractions containing the active compounds were concentrated and submitted to FD-MS.

Known AG Produced by Reference Strains

Five distinct strains were studied producing AG from various groups (gentamicin, kanamycin,

Fig. 1. Ion pair HPLC chromatogram of *M. purpurea* (NRRL 2953) CG-50 active fraction.

Mobile phase MeOH - 0.05 M heptafluorobutyric acid (pH 2.1) (63: 37); flow rate 1.0 ml/minute; sensitivity: Shaded area correspond to active fractions.

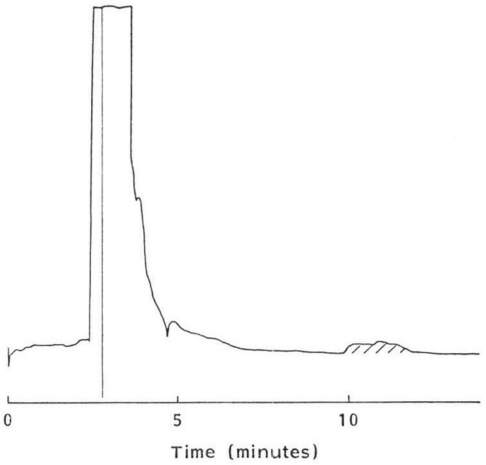
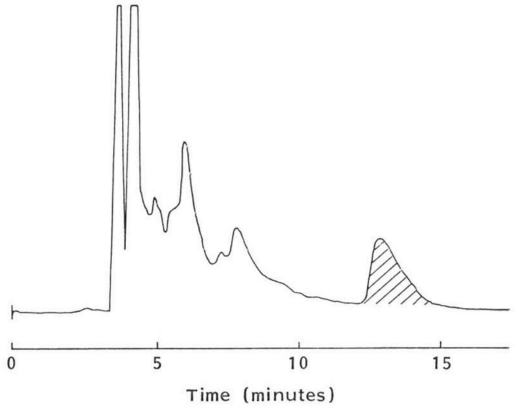


Fig. 2. Ion pair HPLC chromatogram of *S. ribosidificus* (ATCC 21294) CG-50 active fraction.

Mobile phase MeOH - 0.05 M heptafluorobutyric acid (pH 2.1) (57: 43); flow rate 1.0 ml/minute; sensitivity: Shaded area correspond to active fractions.



fortimicin and butirosin).

The results obtained are presented in Table 2. Owing to the differences in productivity of the individual strains, the amount of recovered active material after HPLC varies from 10% (100 μg) to less than 1% (10 μg) of the amount of the injected CG-50 fractions (1 mg). The purification achieved by the HPLC step is thus quite interesting. Figs. 1 and 2 show representative ion pair HPLC chromatograms of *Micromonospora purpurea* and *Streptomyces ribosidificus* CG-50 active fractions.

Reproducible and satisfactory FD mass spectra were obtained for each sample. Scanning of the 200~1,000 daltons mass range failed to detect any contaminant ion, demonstrating the efficiency of the purification process.

The MW obtained for the antibiotics produced by *M. purpurea*[14a,b,c], *Streptomyces kanamyceticus*[15a], *Micromonospora echinospora*[14d,e] and *Micromonospora olivoasterospora*[14f,g] were in complete agreement with published data. It is interesting to note that, in the case of overlapping HPLC peaks such as in *M. echinospora* and *M. purpurea*, the presence of more than one compound was readily detected in the FD mass spectrum. Another solvent system, such as trifluoroacetic acid for the members of the gentamicin family[13], could thus have been selected to resolve the overlapping peaks.

The antibiotic produced by *S. ribosidificus* had a MW corresponding to the expected ribostamycin[14h]. However, it coeluted exactly with xylostatin[25], a stereoisomer of ribostamycin, and not with authentic ribostamycin. Thus, xylostatin appears to be the antibiotic produced. The ability of the ion pair HPLC to distinguish between these two stereoisomers is noteworthy. With the possible exception of xylostatin, the reference strains are then shown to produce the expected aminoglycosides.

AG Produced by Wild Type Strains of Actinomycetes

Six strains were studied; three *Saccharopolyspora* sp. (A1281, A1283 and A1262) and three *Micromonospora* sp. (A874, A1036 and A1038).

Table 3. Characterization by ion pair HPLC/FD-MS of AG produced by wild strains of actinomycetes.

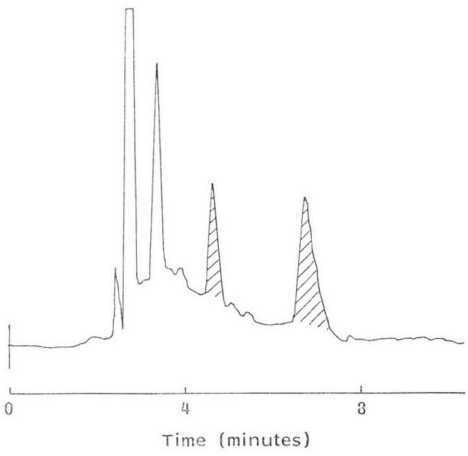
AG producing strains		Microbiological data (susceptible group)	Culture volume (ml)	Weight of CG-50 active fraction (mg)	Factor k′ of active compounds*	Amounts of recovered material (μg)**	FD-MS (m/z) $(M+H)^+$	AG produced	MW
Saccharopolyspora sp.	A1281	Apramycin	60	4	1.2 (a)	>100	540	Apramycin	539
〃	A1283	Apramycin	300	11	1.2 (a)	40	540	Apramycin	539
〃	A1262	Apramycin	300	4	0.8 (a)	40	541	Saccharocin,	540
					1.2 (a)	60	540	apramycin	539
Micromonospora sp.	A1036	Gentamicin	100	3	3.6 (b)	30	450	Gentamicin C_{1a},	449
					6.8 (b)	40	464	gentamicin C_2,	463
					13.5 (b)	20	478	gentamicin C_1	477
〃	A1038	Gentamicin	100	4	0.6 (b)	30	497	Gentamicin B_1 or A_4 or G418,	496
					4 (b)	40	450	gentamicin C_{1a},	449
					7.3 (b)	50	464	gentamicin C_2,	463
					14.5 (b)	50	478	gentamicin C_1	477
〃	A874	Gentamicin	160	7	2.3 (b)	30	448	Sisomicin,	447
					3.5 (b)	50	462	methylsisomicin	461

* (a) Solvent MeOH - 0.05 M heptafluorobutyric acid (pH 2.1) (63: 37); (b) solvent 0.1 M trifluoroacetic acid (1.7).

** Corresponding to a 1 mg injection and estimated from the area under the peak.

Fig. 3. Ion pair HPLC chromatogram of *Saccharopolyspora* sp. A1262 CG-50 active fraction.

Mobile phase MeOH - 0.05 M heptafluorobutyric acid (pH 2.1) (63: 37); flow rate 1 ml/minute; sensitivity: Shaded area correspond to active fractions.



The antibiotics produced by these strains have been characterized as AG on the ground of their antimicrobial activity. Insights into the nature of the antibiotics produced were obtained by microbiological analysis carried directly on culture medium by the agar piece method. Resistant bacteria producing specific AG modifying enzymes[17,18] were used for this analysis (a full account of this analysis will be published elsewhere). According to these data, the antibiotics produced belong either to the apramycin family or to the gentamicin family.

These antibiotics appeared then to be suitable for our study and were analyzed by HPLC/FD-MS. The results obtained are presented in Table 3.

Apramycin was unambiguously shown to be the only antibiotic produced by A1281 and A1283, according to its MW of 539[15b] and by coelution with authentic apramycin. Strain A1262 produced also apramycin together with another antibiotic (Fig. 3) characterized by a MW of 540. This latter product was tentatively identified as saccharocin, a recently discovered aminoglycoside from the apramycin family[19].

Strain A1036 produced the characteristic gentamicin complex: C_{1a} (MW=449, k'=3.6), C_2 (MW=463, k'=6.8) and C_1 (MW=477, k'=13.5[13,14a,b,c]). Strain A1038 produced also the same complex together with a fourth antibiotic more mobile than the three others (k'=0.6) and with a MW of 496. Comparison with published data indicates three possible compounds; gentamicins B_1[14h], A_4[14i] or G418[14j]. A representative ion pair HPLC chromatogram of strain A1038 is shown in Fig. 4.

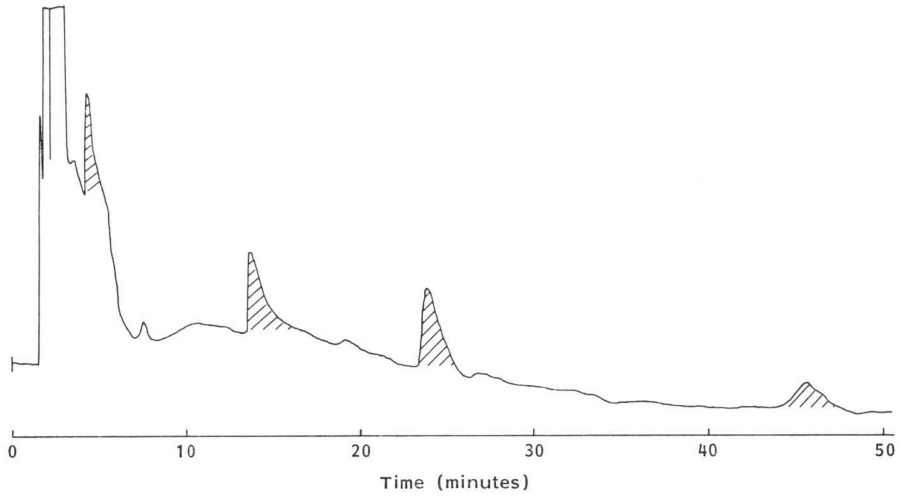
The last strain, A874, gave two active compounds with MW of 447 and 461 respectively. The first one was identified as sisomicin[14d], and the second one as one of the three methylsisomicins (verdamicin I[14e], gentamicin G52[14k] or 1-*N*-methylsisomicin[19c]).

The antibiotics produced by the wild strains have thus been clearly characterized by the HPLC/FD-MS analysis. In some cases, these characterizations have allowed definitive identifications (apramycin, gentamicin, sisomicin). In others, the identifications were not definitive but were narrowed to only a few contenders (saccharocin, methylsisomicin).

These results correlate well with those obtained independently by microbiological analysis which indicated AG from the apramycin and the gentamicin family. This agreement is interesting since the search for possible compounds in data banks was not restricted to AG but extended to other families of antibiotics.

There is also a good agreement in the *Micromonospora* sp. cases with the fact that gentamicins are usually produced by *Micromonospora* species. On the other hand, the results obtained with the *Saccharopolyspora* species are interesting since members of the apramycin family were originally been isolated from *Streptomyces* strains.

Fig. 4. Ion pair HPLC chromatogram of *Micromonospora* sp. A1038 CG-50 active fraction.
Mobile phase 0.1 M trifluoroacetic acid (pH 1.7); flow rate 1 ml/minute; sensitivity: Shaded area correspond to active fractions.



Work is in progress in our laboratory to study new aminoglycosides produced by fermentation using this method.

Discussion

Our results indicate that combination of ion pair HPLC using volatile perfluorinated counter ions and FD-MS provide a reliable characterization of aminoglycosides present in a culture medium through MW determination. In our hands, only 10 μg of purified compound are needed for this analysis and therefore the culture volume can be kept to a minimum. The use of a modern 8 kV mass spectrometer with an adequate signal averaging system[16] should even lower the 10 μg limit by an order of magnitude. This should decrease further the amount of culture volume needed for the analysis. Thanks to the efficiency of the HPLC step, the IRC-50 and CG-50 steps need not to be optimized, resulting in considerable time saving. A whole analysis using this method could be completed within a few hours, although one has to allow time in between for antimicrobial detection.

An important limitation of this method is that, of course, the MW obtained by FD-MS may correspond to several compounds. A new antibiotic may also have the same MW as an already known one. However, since the choice is restricted to at most a few individual products, it should be possible to distinguish among them by considering the retention time (when authentic samples are available) and/or the antimicrobial spectrum. This latter point has a particular significance since it can then be effected on the pure compounds instead of heterogeneous mixtures.

Finally, further progress in this field can be expected with the advent of on line coupling HPLC/MS[20]. On line coupling, often hampered by the presence of non volatile buffers in the eluent, should benefit from the use of volatile counter ions such as perfluorinated carboxylic acids.

Experimental

Apparatus and Reagents

The following instruments were used in this study: High pressure liquid chromatograph; a Waters M6000A pump, equipped with a U6K Liquid chromatography injector (Waters Ass. Milford, MA, USA), a Jobin Yvon refractive index (RI) detector (Iota Instruments S.A. Jobin Yvon, France) and an analytical reverse phase (5 μm, C18) column Ultrasphere Ion Pair, 25 cm $\times$ 4.6 mm (Altex Scientific,

Palo Alto CA, USA) connected with an in-line guard column RP18 spheri 5 (Browlee Labs.), alternatively a PRP 1 Hamilton column, 25 cm×4.6 mm (Reno, Nevada, USA) was used, resolution was lower than with the C18 column but stability in acidic conditions was better (specially important when trifluoroacetic acid is used), mass spectrometer; Varian 311A (Bremmen, Fed. Germany) and purchased emitters from Linden Chromaspec (Bremmen Fed. Germany); Sorvall superspeed RC2-B.

Several reagents were employed. Heptafluorobutyric acid and trifluoroacetic acid were purchased from Fluka (CH. 94-70 Buchs, Switzerland) and were used without further purification. MeOH and H_2O were HPLC grade. AG antibiotics were kindly provided by G. ETIENNE (Toulouse University, France). Lividomycin was obtained from Roger Bellon Laboratories (Toulouse, France); fortimicins from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan); gentamicins and sisomicin from Cetrane Laboratories (Levallois-Perret, France); verdamicin and gentamicin (G418) from Schering Corporation (Bloomfield, USA); kanamycin and streptomycin from Rhone-Poulenc Laboratories (Vitry, France); butirosin form Parke Davis and Co. (St Louis, MO, USA); xylostatin from Takeda Chemical Industries, Ltd. (Osaka, Japan); ribostamycin from Delalande (Courbevoie, France). Amberlite CG-50 and IRC-50 resins were purchased from SERVA Laboratoriums and BDH Chemicals Ltd. (England).

Microorganisms, Antibacterial Activity and Antibiotics Production

Assay Bacteria: *Bacillus subtilis* ATCC 6633. The strain was grown for 2 days at 37°C on a sporulation medium consisting of Nutrient broth (Difco) 8 g, yeast extract 4 g, $MnCl_2 \cdot 4H_2O$ 10 mg, agar 15 g, distilled water 1 liter, adjusted to pH 7.2 before autoclaving. The spores were collected in physiologic water, heated at 70°C for 30 minutes and stored at 4°C. The assay medium consisted of Polypeptone (Biokar) 5 g, beef extract (Difco) 3 g, TAPS (Sigma) 3 g, agar 20 g, distilled water 1 liter. The medium was adjusted to pH 9 before autoclaving then inoculated with 1,000 cfu/ml of *B. subtilis* and incubated for 18 hours at 37°C.

Aminoglycoside-producing Strains

The following procedures are representative.

Production of Antibiotic on Solid Medium: The strains are grown for 10 to 14 days at 28°C on a medium (M151) consisting of starch 3%, soybean meal 1.5%, yeast extract 1%, glycerol 3%, $FeSO_4 \cdot 7H_2O$ 0.01%, $CaCO_3$ 0.5%, agar 1.5% and distilled water for 1 liter (autoclaved 20 minutes at 120°C).

Production of Antibiotic on Liquid Medium: Spores grown for 10 to 15 days at 28°C on ATCC medium 172 are inoculated in a seed medium (M221) consisting of starch 1%, glucose 1%, peptone 0.5%, beef extract 0.2%, yeast extract 0.3%, soybean meal 0.2%, $CaCO_3$ 0.2%, distilled water for 1 liter. After 2~3 days at 28°C on a rotary shaker (220 rpm) one of the two following media are inoculated with the seed medium in the ration 3~5%: Medium 254; starch 4%, soy peptone 0.5%, yeast extract 4%, K_2HPO_4 0.2%, NaCl 0.2%, $MgSO_4 \cdot 7H_2O$ 0.2%, $CaCO_3$ 0.1%. Medium M222; same as M151 without agar. After 3 to 5 days at 28°C on a rotary shaker (220 rpm), the broths are collected.

The culture are then tested for antimicrobial activity.

Extraction of the Antibiotics

The following procedures are representative.

AG Produced on Solid Medium: 30 ml of agar medium and 30 ml of 0.1 N aq HCl are homogenized for 5 minutes in a Waring blender. The resulting mixture is then centrifuged and the supernatant is collected. The pellet is extracted a second time with 30 ml of 0.05 N HCl, the two supernatants are combined and oxalic acid (1 g) is added to eliminate calcium ions. The solution is filtered and neutralized with aq NH_4OH.

AG Produced on Liquid Medium: The pH of the culture broth is adjusted to 2 with 1 N HCl. The mixture is agitated for 1 hour at ambient temp, centrifuged and the supernatant collected and neutralized with aq NH_4OH.

Purification of the Antibiotics

The extract previously obtained is applied to the top of a short IRC-50 column (NH_4^+). The

column is washed with H_2O and eluted with 2 N NH_4OH. Fractions are collected and tested for antimicrobial activity against *B. subtilis*. The active fractions are then concentrated under reduced pressure and the residue is applied to the top of a short CG-50 column (NH_4^+) and the column developed with a gradient of NH_4OH (0→2 N). The active fractions are concentrated to dryness under reduced pressure and weighted.

Ion Pair HPLC of the Active Fraction

About 1 mg of the collected fraction is injected into a C18 or a PRP1 HPLC column. Flow rate was set at 1 ml/minute throughout this work. All detections were performed using RI detector. The exact composition of the mobile phase used for each strain studied is given in Tables 2 and 3. In case of completely unknown compounds, a gradient HPLC is first performed (MeOH - 0.05 M heptafluorobutyric acid, 40: 60~90: 10). Fractions are collected and tested for antimicrobial activity. Comparison of retention time with those of known compounds allows determination of an adequate isocratic mobile phase. After HPLC, the different components separated are collected and tested for antimicrobial activity. The active fractions are concentrated to dryness under reduced pressure to afford pure AG.

FD-MS

The pure compounds previously collected (10~100 μg) are dissolved in 3 to 10 μl of MeOH - H_2O (1: 3, 2: 3). 0.5 μl of this solution is deposited on the emitter. Signals are observed with emitter currents of 15 to 20 mA.

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References

1) LITTLETON, M. A.: A.M.A. Drug Evaluation. 4th Ed., Am. Med. Soc. Clin. Sci. Group, pp. 767~768, 803~804, 963~964, Chicago, 1980
2) NARA, T.; M. YAMAMOTO, I. KAWAMOTO, K. TAKAYAMA, R. OKACHI, S. TAKASAWA, T. SATO & S. SATO: Fortimicins A and B, new aminoglycoside antibiotics. I. Producing organism, fermentation and biological properties of fortimicins. J. Antibiotics 30: 533~540, 1977
3) BU' LOCK, J. D.; L. J. NISBET & D. J. WINSTANLEY: Bioactive Microbial Products: Search and Discovery. The Society for General Microbiology. Academic Press, 1982
4) ETIENNE, G.: Méthodes pour la sélection de *Streptomyces* producteurs d'antibiotiques de la famille des aminoglycosides. Thèse de Spécialité, Toulouse Université, France, 1980
5) HOTTA, K.; A. TAKAHASHI, Y. OKAMI & H. UMEZAWA: Relationship between antibiotic resistance and antibiotic productivity in actinomycetes which produce aminoglycoside antibiotics. J. Antibiotics 36: 1789~1791, 1983
6) CLAES, P. J. & H. VANDERHAEGHE: Thin layer chromatography identification of aminoglycoside antibiotics. J. Chromatogr. 248: 483~487, 1982
7) RINEHART, K. L., Jr. & J. C. COOK, Jr.: Field desorption mass spectra of antibiotics. J. Antibiotics 27: 1~13, 1974
8) SCHULTEN, H. R.: Off-line combination of liquid chromatography and field desorption mass spectrometry: Principles and environnemental, medical and pharmaceutical applications. J. Chromatogr. 251: 105~128, 1982
9) DIXON, D. J.: Application du couplage direct de la chromatographie en phase liquide et de la spectrométrie de masse aux problèmes biochimiques. Analysis 10: 343~348, 1982
10) RINEHART, K. L.: Structure elucidation of antibiotics. The future of antibiotherapy and antibiotic research. Proceedings of the 2nd Rhone-Poulenc Table Conference. pp. 437~448, Academic Press, New York, 1981
11) WHITE, E. R. & J. E. ZAREMBO: Reverse phase high speed liquid chromatography of antibiotics. III.

Use of ultra high performance columns and ion-pairing techniques. J. Antibiotics 34: 836~844, 1981
12) WHALL, T. J.: Determination of streptomycin sulfate and dihydrostreptomycin sulfate by high performance liquid chromatography. J. Chromatogr. 219: 89~100, 1981
13) INCHAUPÉ, G. & D. SAMAIN: Use of perfluorinated carboxylic acids in the separation of aminoglycoside antibiotics by ion-pair reverse phase high performance liquid chromatography. J. Chromatogr. 303: 277~282, 1984
14) UMEZAWA, H.: Index of Antibiotics from Actinomycetes. Vol. II. a: p. 423, b: p. 424, c: p. 425, d: p. 937, e: 1030, f: p. 393, g: p. 394, h: p. 422, i: p. 420, j: p. 117, k: p. 1169, Jpn. Sci. Soc. Press, Tokyo, 1978
15) BERDY, J.; A. ASZALOS, M. BOSTIAN & K. MC. NITT: Handbook of Antibiotic Compounds. Vol. I. Carbohydrate Antibiotics. a: p. 160, b: p. 207, c: p. 188, CRC Press, Inc, Boca Raton, Florida, 1978
16) SNELLING, C. R., Jr.; J. C. COOK, R. M. MILBERG, M. E. HEMLING & K. L. RINEHART, Jr.: Minicomputer-based multichannel signal averager for acquisition of weak and transient mass spectra. Anal. Chem. 56: 1474~1481, 1984
17) DAVIES, J. & D. I. SMITH: Plasmid determined resistance of antimicrobial agents. Ann. Rev. Microbiol. 32: 469~518, 1978
18) UMEZAWA, H. & S. KONDO: Handbook of Experimental Pharmacology. pp. 1~62, Springer Verlag, Berlin, 1982
19) AWATA, M.; S. SATOI, N. MUTO, M. HAYASHI, H. SAGAI & H. SAKAKIBARA: Saccharocin, a new aminoglycoside antibiotic. Fermentation, isolation, characterization and structural study. J. Antibiotics 36: 651~655, 1983
20) ARPINO, P. J.: Chromatography combined with mass spectrometry. Mass Spectrometry Advances, A. pp. 161~172, 1982
21) WAGMAN, G. H.; R. T. TESTA & J. A. MARQUEZ: Antibiotic 6640. II. Fermentation, isolation, and properties. J. Antibiotics 23: 555~558, 1970
22) UMEZAWA, H.; M. UEDA, K. MAEDA, K. YAGISHITA, S. KONDŌ, Y. OKAMI, R. UTAHARA, Y. ŌSATO, K. NITTA & T. TAKEUCHI: Production and isolation of a new antibiotic, kanamycin. J. Antibiotics, Ser A 10: 181~188, 1957
23) WEINSTEIN, M. J.; G. M. LEUDEMANN, E. M. ODEN & G. H. WAGMAN: Gentamicin, a new broad-spectrum antibiotic complex. Antimicrob. Agents Chemother. -1963: 1~7, 1964
24) SHOMURA, T.; N. EZAKI, T. TSURUOKA, T. NIWA, E. AKITA & T. NIIDA: Studies on antibiotic SF-733, a new antibiotic. I. Taxonomy, isolation and characterization. J. Antibiotics 23: 155~161, 1970
25) HORII, S.; I. NOGAMI, N. MIZOKAMI, Y. ARAI & M. YONEDA: New antibiotic produced by bacteria, 5-β-D-xylofuranosylneamine. Antimicrob. Agents Chemother. 5: 578~581, 1974