RIT 2214, A NEW BIOSYNTHETIC PENICILLIN PRODUCED BY A MUTANT OF *CEPHALOSPORIUM ACREMONIUM*

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A number of lysine-requiring auxotrophs of *Cephalosporium acremonium* were investigated for incorporation of side-chain precursors and for accumulation of β-lactam compounds. One of the auxotrophs, *Acremonium chrysogenum* ATCC 20389, producing cephalosporin C and penicillin N only if grown in media supplemented with DL-α-amino-adipic acid (DL-α-AAA), was found to use L-S-carboxymethylcysteine (L-CMC) as a side-chain precursor for the synthesis of a new penicillin (RIT 2214). No corresponding cephalosporin was detected. The penicillin present in the culture filtrate, was concentrated by adsorption on activated carbon and successive column chromatography on Amberlite IRA-68 and Amberlite XAD-4. Final purification was achieved by cellulose column chromatography.

RIT 2214 was identified as 6-(D)-{[(2-amino-2-carboxy)-ethylthio]-acetamido}-penicillanic acid by spectral analysis, bioactivity spectrum, elucidation of side-chain structure and finally by semisynthesis. Its biological properties were also evaluated.

Typically, *Penicillium chrysogenum* synthesizes a large variety of N-acyl penicillin compounds by the method of precursor supplementation. This is in marked contrast to the few β-lactam antibiotics, all containing the D-α-amino-adipyl side-chain, produced by *Cephalosporium acremonium*[1]. Hitherto described penicillin N producing species are insensitive to incorporation of extraneous side-chain precursors into antibiotic. This study was designed to evaluate the incorporation of penicillin precursors and/or α-AAA analogues by a lysine-requiring mutant of *Cephalosporium acremonium*, blocked in its lysine pathway before α-aminoadipate.

The present communication describes the production, isolation, elucidation of the chemical structure and biological characterization of a new penicillin synthesized by L-S-carboxymethylcysteine (L-CMC) feeding of the mutant culture.

Possible consequences on the hypothetical branched pathway for biosynthesis of penicillin N and cephalosporin C are discussed.

Material and Methods

Strains and Culture Conditions

The RIT 2214 producing lysine auxotroph, *Acremonium chrysogenum* ATCC 20389, was derived from the original wild type *Cephalosporium* sp. BROTZU strain ATCC 11550 (reclassified as *Acremonium chrysogenum*[2]) by treatment with N-methyl-N′-nitro-N-nitrosoguanidine (ROELANTS, unpublished results, 1971).

Morphologic characteristics of the mutant strain were described previously[3]. Cultures were maintained on complete sporulation medium.

Sporulation medium: Soya-bean meal, 15 g; $CaCO_3$, 10 g; glucose, 10 g; sucrose, 36 g; agar, 20 g; 1,000 ml deionized water, pH adjusted to 7.0. Inoculated slants were incubated at 28°C for 12 days followed by storage at 4°C.

Fermentation Procedures

A 1-ml suspension of spores, obtained from one slant, was used to inoculate flasks containing seed medium.

Penicillin precursors[4]: Phenylacetic acid, phenylacetamide, phenoxyacetic acid, cyclohexylacetic acid, propionic acid, butyric acid, hexanoic acid, *trans*-2-hexenoic acid, adipic acid, methylmercaptoacetic acid, *n*-butylmercaptoacetic acid.

α-AAA analogues: D- and DL-α-aminoadipic acid[5], DL-2-bromoadipic acid[5], DL-2-azidoadipic acid[6], DL-2, 5-diaminoadipic acid[7], DL-piperidonecarboxylic acid[8], DL-glutamic acid, DL-α-aminopimelic acid, L- and D-S-carboxymethylcysteine[9], DL-3, 3-dimethyl-S-carboxymethylcysteine[9], L-5-methyl-S-carboxymethylcysteine[9], L-S-carboxymethylcysteine-sulfoxide[10], L-S-carboxymethylcysteine-sulfon[11]. Side-chain precursor solutions were filter-sterilized and added to the inoculated fermentor medium after 24-hour incubation.

Detection of Antibiotics

The precursor supplemented fermentor medium was screened microbiologically for the presence of β-lactam antibiotics using *Alcaligenes faecalis* ATCC 8750, *Alcaligenes viscolactis*, *Escherichia coli* ATCC 10536, and *Staphylococcus aureus* R1001 (cephalosporin P resistant strain, BOON and ROELANTS unpublished results, 1969) as indicator organisms. Discrimination between penicillin and cephalosporin compounds was made by incorporation of 50 units/ml penicillinase in the assay-medium. Cephalosporin C activity was determined qualitatively by a plate assay method, using *Alcaligenes faecalis* ATCC 8750[12,13] in agar containing penicillinase. *Alcaligenes viscolactis* (RIT departmental strain) was found to be about ten-times as sensitive to penicillin N as to cephalosporin C. Hence this organism was suitable for detection and qualitative determination of penicillin N-like antibiotics.

Chromatographic Separation of β-Lactam Antibiotics

Chromatography was performed to confirm the production of penicillin-like and/or cephalosporin-like compounds by the original wild type strain and the biochemical mutant.

Filtered broth samples were spotted onto Whatman no. 4 paper. Chromatograms were developed with *n*-butanol-acetic acid-water (4: 1: 5) upper phase. The β-lactam antibiotics were detected by bioautography on respectively *Bacillus subtilis* ATCC 6633; *Alcaligenes faecalis* ATCC 8750 and *Alcaligenes viscolactis*-seeded agar plates. Antibiotics were identified by Rf and co-chromatography of standards. 6-APA and/or 7-ACA production was evaluated chromatographically by a well-known procedure[14].

Synthesis of 6-D-{[(2-Amino-2-carboxy)-ethylthio]-acetamido}-penicillanic acid (III)

To a stirred solution of 3.63 g (0.03 mole) of D-cysteine (**I**) and 2.4 g (0.03 mole) of ammonium bicarbonate in 100 ml of distilled water flushed with nitrogen, was added at once, 11.25 g (0.03 mole) of 6-(α-bromoacetamido)-penicillanic acid ammonium salt (**II**) prepared according to PERRON *et al.*[15] Stirring was continued at room temperature for 30 minutes. The mixture was then cooled to 5°C, layered with 100 ml of methylisobutylketone (MIBK) and extracted by mixing vigorously while adjusting the pH at 3.0 with Dowex-50×12 (H^+). This extraction procedure was repeated twice and MIBK extracts were discarded. The aqueous phase was carefully neutralized with 25 % ammonium hydroxide solution and concentrated up to 50 ml by evaporation under reduced pressure at 30°C.

The concentrate was poured in 500 ml ice-cold ethanol. The precipitate that formed was collected by filtration, washed with 50 ml cold ethanol and dried *in vacuo* over P_2O_5 to yield 11.0 g of compound **III**. An analytical sample of compound **III** was prepared by dry-column chromatography[16] on microcrystalline cellulose. The column was developed with an isopropanol - water (70: 30) mixture and was then sliced in 2-cm thick segments which were eluted with 20 ml distilled water. The purified antibiotic was detected by spotting 50 μl amounts of different fractions on thin-layer silica gel plates. The thin-layers were developed with the following solvent systems: *n*-butanol - ethanol - acetic acid - water (10: 1.5: 1.5: 2)[17]; *n*-butanol - acetic acid - water (4: 1: 1)[18] and acetonitrile - water (75: 25)[19].

The purified antibiotic was identified on the plates by spraying with respectively: ninhydrin; 0.01 N I_2/starch; 1 N NaOH/0.02 N I_2/starch and finally by bioautography on *Bacillus subtilis* ATCC 6633-seeded agar plates. Pure antibiotic containing fractions were combined and lyophilized to give

pure compound **III**. Purity of this penicillin (87%) was assessed according to the hydroxamic acid assay-method[20] with crystalline penicillin G as reference compound.

Analytical Methods

The IR spectra were obtained from KBr discs by means of a Beckman IR-4 infrared spectrometer. Nuclear magnetic resonance spectra were recorded with a Perkin Elmer R-12 (60 MHz) spectrometer on 15% solutions of the compounds in D_2O with tetramethylsilane as reference compound. Optical rotation measurement was made with a Perkin Elmer Model 141 polarimeter.

Materials

Penicillin precursors, DL-glutamic acid, DL-α-aminopimelic acid, L-lysine and Dowex W 50×12 (20~50 mesh, H^+) were purchased from Fluka (Buchs, Switzerland). D- and L-cysteine, D-penicillamine were obtained from Diamalt (Munich, BRD). D-α-Aminoadipic acid and penicillin N were a generous gift from Prof. H. VANDERHAEGHE (Rega Institute, Leuven, Belgium). Penicillinase (staphylococcal origin) was purchased from Leo Pharmaceutical Products (Ballerup, Denmark). Amberlite IRA-68 and Amberlite XAD-4 were supplied by Rohm & Haas (Philadelphia, USA). Cephalosporin C was provided by Glaxo Laboratories Ltd. (Greenford, UK). Microcrystalline cellulose was obtained from Mackerey Nagel & Co. (Düren, BRD). Silica gel TLC-Ready Plastic Sheets (20×20 cm) F1500 LS 254 were obtained from Schleicher & Schüll (Dassel, BRD).

Results

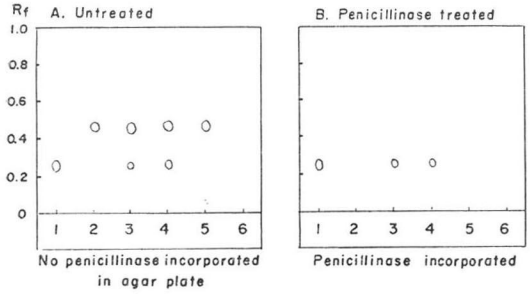
1. Spectrum of Antibiotics Produced by *Acremonium chrysogenum* ATCC 20389

The lysine-requiring auxotroph grew well when DL-α-AAA or L-lysine was added to the culture media. Penicillin N and cephalosporin C production was only obtained with DL-α-AAA incorporation in the fermentor medium. The α-AAA analogue, L-S-carboxymethylcysteine induced the formation of a penicillin N-like compound (Table 1 and Fig. 1 A and B). No corresponding cephalosporin was detected. No such penicillin activity was formed when other side-chain precursors were supplemented, nor was there any trace of 6-APA and/or 7-ACA production. There was also no evidence for synthesis of deacetoxy- and/or deacetylcephalosporin C-like compounds.

Fig. 1. Bioautographic evidence for synthesis of penicillin N-like compound (RIT 2214) by L-CMC supplementation of *Acremonium chrysogenum* ATCC 20389 culture.

(1) authentic cephalosporin C; (2) authentic penicillin N; (3) culture broth of wild type strain ; (4) DL-α-AAA supplemented culture broth of mutant; (5) L-CMC supplemented culture broth of mutant; (6) unsupplemented culture broth of mutant.

Support : Whatman no. 4
Mode : descending
Solvent : *n*-butanol - acetic acid - water (4: 1: 5)
Bioactivities were detected by bioautography on *Bacillus subtilis* ATCC 6633 seeded agar. Penicillinase incorporated in agar plate B.



2. Time Course of β-Lactam Antibiotic Accumulation by *Acremonium chrysogenum* ATCC 20389 and Parent Wild type Strain

In contrast with simultaneous penicillin N and cephalosporin C production by the wild type strain (Fig. 2A) we found a 20-hour time-delay during fermentation of both antibiotics by the lysine auxotroph (Fig. 2B) and observed that penicillin N production started first.

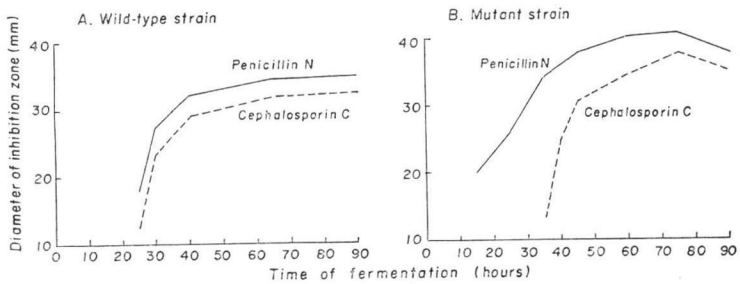
3. Isolation and Purification of RIT 2214

The 3-day, L-CMC supplemented culture broth (*ca.* 12 liters) was filtered. The 10 liters

Table 1. Bio-assay of α-AAA or L-CMC supplemented *Acremonium chrysogenum* ATCC 20389 culture broths.

Precursors	Test organisms and diameter of inhibition zones (mm)							
	Alcaligenes faecalis ATCC 8750	*Alcaligenes faecalis* +penicillinase	*Alcaligenes viscolactis*	*Alcaligenes viscolactis* +penicillinase	*Escherichia coli* ATCC 10536	*Escherichia coli* +penicillinase	*Staphylococcus aureus* R 1001	*Staphylococcus aureus* +penicillinase
DL-α-AAA	28	27	32	15	22	0	23	0
L-CMC	18	0	35	0	24	0	28	0

Fig. 2. β-Lactam fermentation profile for parental wild type strain (A) and mutant strain (B).
The mutant fermentor medium was DL-α-AAA supplemented (2 g/liter).
Alcaligenes faecalis ATCC 8750 and *Alcaligenes viscolactis* were used as test organisms for the bioassay of respectively cephalosporin C and penicillin N.



filtrate was cooled to 5°C, mixed for 45 minutes with 300 g activated carbon while the pH was continuously adjusted at 4.0 with 1 N HCl. The charcoal was filtered with the aid of diatomaceous earth, washed with distilled water and then eluted with 9.0 liters of a 60: 40 acetone-water mixture containing sufficient NH_4OH to maintain the pH of the eluate at 6.5. The eluate was concentrated to 500 ml *in vacuo* (30°C) and then poured on a IRA–68 column (8.0×42 cm, formate cycle). After the column was washed with distilled water, RIT 2214 was eluted with 0.2 M ammonium formate buffer (pH 6.5). Antibiotically active fractions (250 ml) were pooled (total volume: 3.6 liters) and then concentrated to 150 ml *in vacuo*. The concentrate was applied on a XAD–4 column (4.0×80 cm) and the antibiotic was eluted with distilled water. Fractions were monitored for conductivity and analyzed for bioactivity. The antibiotically most active

Table 2. Rf-values of RIT 2214 as compared with other β-lactam antibiotics.

	T.L.C.			P.C.	
	S_1	S_2	S_3	SI	SII
RIT 2214	65	72	22	43	9
Penicillin N	65	72	22	43	9
Cephalosporin C	65	72	22	25	9
Ampicillin	—	—	—	69	44
Penicillin G	88	87	73	—	—
6-APA	—	—	38	—	—
7-ACA	—	—	42	—	

TLC
Support : Silica gel
Mode : Ascending
Solvents:
S_1 *n*-butanol - acetic acid - water (4: 1: 1)
S_2 *n*-butanol - water - ethanol - acetic acid (5: 2: 1.5: 1.5)
S_3 acetonitrile - water (75: 25)
Detection: Ninhydrin
Iodized starch
Bioautography on *B. subtilis* ATCC 6633 seeded agar

PC
Support: Whatman no. 4
Mode : Descending
Solvents:
SI *n*-butanol - acetic acid - water (4: 1: 5)
SII *n*-butanol - ethanol - water (4: 1: 5)
Detection: Bioautography on *B. subtilis* ATCC 6633 seeded agar

Fig. 3. I.R. spectra of authentic D-CMC and D-CMC isolated after acid hydrolysis of RIT 2214.

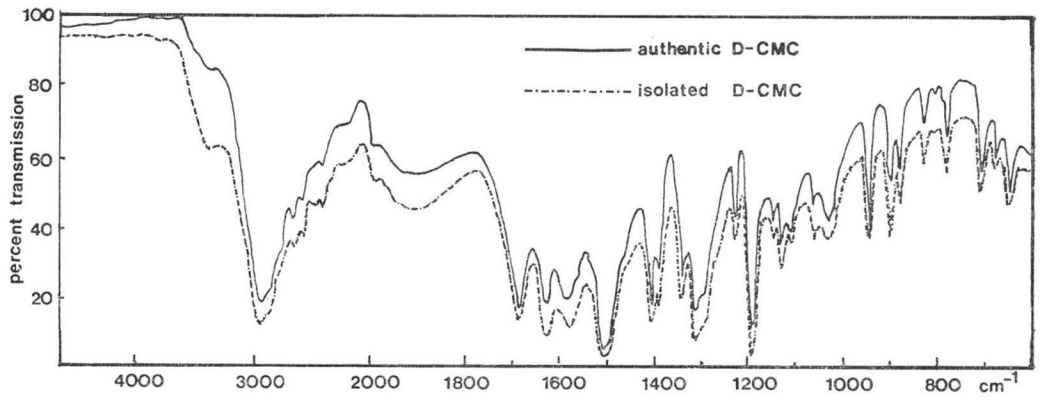


Fig. 4. I.R. spectrum of RIT 2214 (KBr).

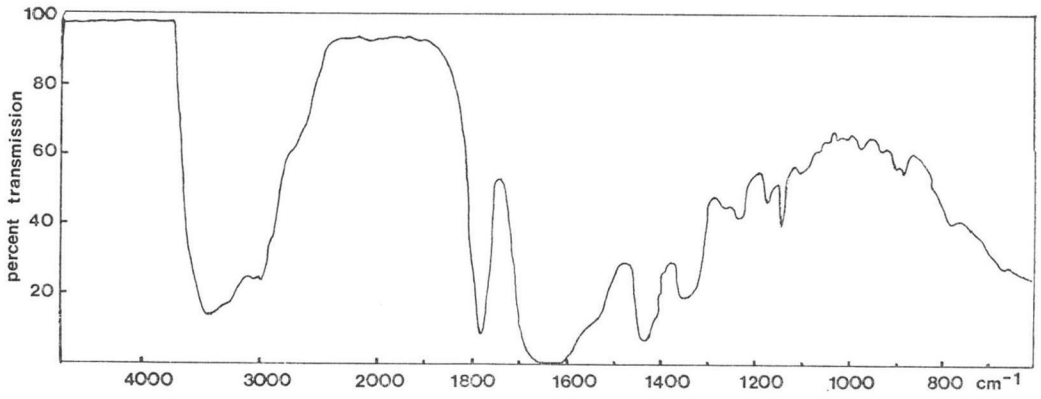


Fig. 5. N.M.R. spectrum of RIT 2214 in D_2O.

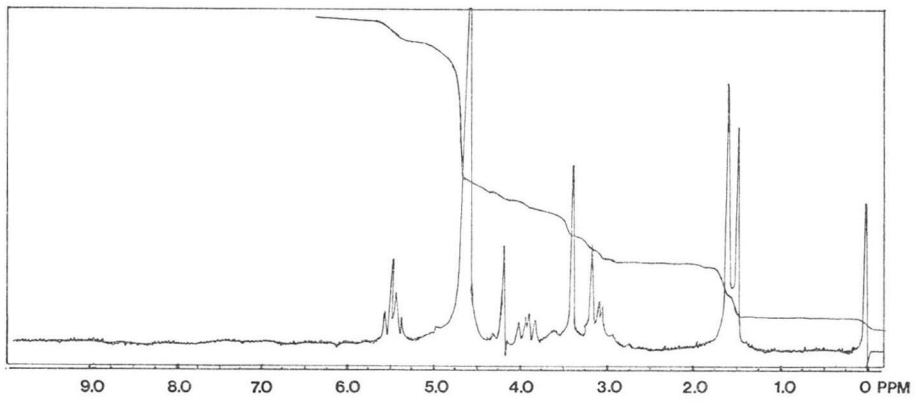


Fig. 6. Synthesis of compound III.

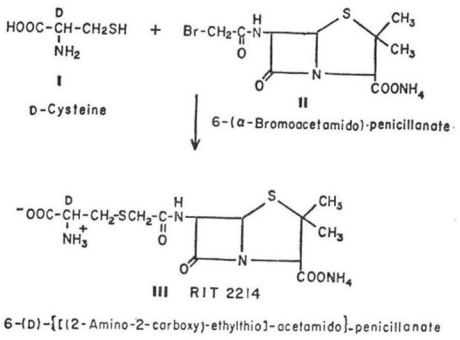
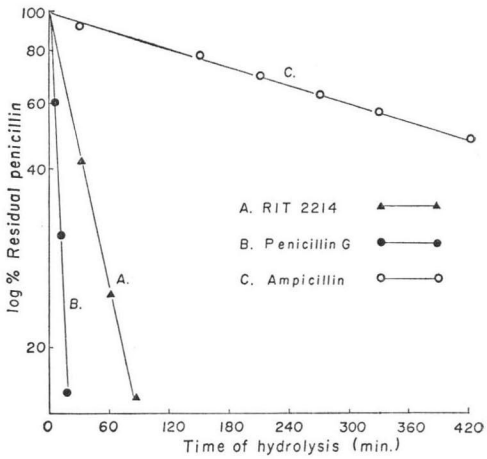


Fig. 7. Stability of RIT 2214, penicillin G and ampicillin in synthetic gastric juice. Composition of buffer: NaCl, 2 g; HCl 1 N, 80 ml; pepsin, 3.2 g; distilled water to 1 liter, pH 1.3 at 37°C.



fractions were combined and concentrated under reduced pressure by azeotropic distillation with isopropanol to give 330 mg of a yellow powder. Finally, RIT 2214 was further purified according to a dry-column chromatography technique on cellulose (as described elsewhere in this paper) and recovered as a off-white powder. This material was about 70% pure.

4. Identification of RIT 2214

Like penicillin N, RIT 2214 was positive to ninhydrin, iodized starch and NaN_3–I_2 and it was also destroyed by penicillinase.

Penicillin N and RIT 2214 were found to be chromatographically indistinguishable as shown in Table 2.

RIT 2214 (800 mg) was hydrolyzed in N HCl for 8 hours at 100°C under N_2. The hydrolysate was evaporated several times to dryness *in vacuo* with intermittent addition of distilled water. The residue was chromatographed on Dowex 1×8 (OH^-, 100~200 mesh) column (2.0×40 cm). The column was washed with distilled water and then with 0.1 N CH_3COOH. Acidic amino acids were eluted with 1 N CH_3COOH, the eluate being collected in 20-ml fractions. The rate of flow was adjusted to 1 ml/min. The S-carboxymethylcysteine containing fractions were located by spotting samples (10 μl) of each fraction on a silica gel thin-layer plate, spraying with ninhydrin solution and then heating at 100°C for 10 minutes. Pure CMC containing fractions (13~20, assessed with TLC on silica gel, solvent system *n*-butanol-acetic acid-water; 4: 1: 1) were pooled and evaporated to dryness *in vacuo*. The residue was dissolved in 10 ml of water, then 10 ml of absolute ethanol was added and the pH of this solution was adjusted at 2.9 with a few drops of 25% NH_4OH. The amino acid crystallized overnight at 4°C. The substance had m. p. 195°~196°C and the mixed m. p. with an authentic sample of D-S-carboxymethylcysteine was not depressed. Its optical configuration was $[\alpha]_{436}^{25}$ −0.040 (*c* 5.5 mg/ml, 1 N HCl). Authentic L-CMC showed under identical conditions an $[\alpha]_{436}^{25}$ +0.038 value. IR-spectra of isolated D-CMC and an authentic sample of D-CMC were fully superimposable (Fig. 3). The IR-spectrum of RIT 2214 showed the characteristic β-lactam carbonyl absorption at 1780 cm^{-1} (Fig. 4). Its NMR-spectrum (Fig. 5) revealed the existence of signals at 3.15 (2H, m), 3.40 (2H, s) and 3.90 (1H, m) ppm which were assigned to the S-carboxymethylcysteine side-chain.

From the above data, RIT 2214 was presumed to be a penicillin N analogue with D-CMC as side

Table 3. Antibacterial spectrum of RIT 2214 and ampicillin.

Test organism	MIC (μg/ml)	
	RIT 2214	Ampicillin
Corynebacterium xerosis	0.1	0.1
Staphylococcus aureus	1.2	1.2
Staphylococcus aureus Penicillin-resistant	>80	20
Bacillus subtilis	0.3	0.3
Escherichia coli	1.2	1.2
Escherichia coli Penicillin-resistant	>80	>80
Aerobacter aerogenes	1.2	1.2
Enterobacter cloacae	20	1.2
Klebsiella pneumoniae	>80	40
Salmonella typhi	0.6	0.6
Salmonella paratyphi	1.2	1.2
Salmonella typhimurium	40	40
Salmonella meleagridis	0.6	0.6
Salmonella panama	40	2.5
Salmonella anatum	0.6	0.6
Vibrio El Tor	0.6	1.2
Brucella abortus	20	0.6
Bordetella bronchiseptica	0.6	0.6
Shigella sonnei	20	2.5
Proteus morganii	40	20
Proteus mirabilis	1.2	1.2
Pseudomonas aeruginosa	>80	>80

Table 4. *In vivo* activity of RIT 2214 in mice.

Pathogens	PD_{50} (mg/kg)	
	RIT 2214	Ampicillin
Staphylococcus aureus No. 663	<10	<10
Escherichia coli No. 47	<10	23.6
Escherichia coli No. 9	11	11.5
Escherichia coli SKF 12140	37.2	56.3
Salmonella enteritidis No. 24	13.5	55.6
Salmonella brandenburg No.22	13	36.4
Salmonella panama No. 20	<10	22
Shigella flexneri No. 25	52.5	>100
Proteus mirabilis No. 11	<10	14.8
Klebsiella pneumoniae SKF 4200	<10	36.7

chain instead of D-α-AAA. Final identification was achieved by comparison of RIT 2214 with compound **III** synthesized from **II** (Fig. 6). RIT 2214 was essentially identical with compound **III** in IR-, NMR-spectra and chromatographic behaviour and consequently, RIT 2214 was identified as 6-(D)-{[(2-amino-2-carboxy)-ethylthio]-acetamido}-penicillanic acid.

5. Stability of RIT 2214 in Acidic Buffer

Appropriate amount of penicillins: RIT 2214, penicillin G, and ampicillin were dissolved in pH 1.3 synthetic gastric juice (for composition see Fig. 7) which had previously been brought to 37°C. Initial concentration of the drugs was adjusted to 1×10^{-3} M. The reaction was carried out in duplicate in a volumetric flask which was immersed in constant temperature bath kept at 37°C. Samples were taken at intervals and assayed immediately for residual antibiotic content according to the hydroxamic acid method.

6. Biological Activity of RIT 2214 — Antibacterial Spectrum

The minimum inhibitory concentrations (MIC) of RIT 2214 and ampicillin were determined against a wide variety of bacteria by the two-fold tube dilution technique using 9-ml volumes of nutrient broth inoculated with 0.05 ml of 1/100 dilution of overnight grown culture; tubes were read after 10 hours incubation at 37°C. The results are shown in Table 3 together with those for ampicillin which was tested for comparison. RIT 2214 was observed to exhibit broad spectrum antibacterial activity. *Salmonellae* species were found to be very sensitive to this penicillin. Ampicillin however was more potent than RIT 2214 *in vitro*. RIT 2214 showed no activity against penicillin-resistant organisms.

7. *In Vivo* Activity

RIT 2214 was evaluated against ampicillin in experimental infections of mice. Mice were challenged intravenously with the pathogens suspended in 9‰ saline. A single subcutaneous treatment with the antibiotic was given 1 hour after bacterial challenge. Groups of 10 mice were used for each dosage level (100, 33 and 11 mg/kg) and the animals observed for 5 days to determine the median protective dose

(PD_{50})[21]. The results of the *in vivo* experiments are shown in Table 4.

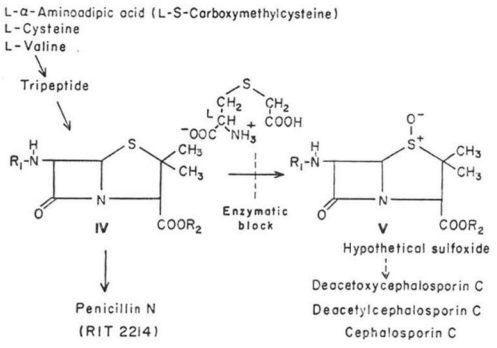
Surprisingly, RIT 2214 afforded better protection than ampicillin against most of the infections tested.

Discussion

LEMKE and NASH[22] scrutinized mutant strains of *Cephalosporium acremonium* for production of β-lactam antibiotics. They obtained a lysine auxotroph unable to synthesize either penicillin N or cephalosporin C. This mutant grew in a minimal medium supplemented with lysine but not with α-aminoadipic acid. Supplementation of the mutant culture with both lysine and α-AAA resulted in the synthesis of trace amount of antibiotic. The lysine auxotroph we studied seems to be blocked before α-aminoadipate since it growths on media either α-AAA or lysine supplemented. No antibiotic activity is detected when this mutant is propagated in a medium only enriched with lysine. The presence of exogenous α-AAA in the medium is sufficient for both growth and substantial production of penicillin N and cephalosporin C. Side-chain precursor incorporation into penicillin by *Penicillium chrysogenum* has been reported to be unspecific.

This is not the case for *Cephalosporium acremonium*. Indeed only the α-AAA analogue, L-S-carboxymethylcysteine is incorporated into penicillin N-like antibiotic (RIT 2214) by our mutant. The reason why no corresponding cephalosporin is synthesized remains puzzling. STEVENS[23] observed a decrease in the production of cephalosporin C but an increase in that of penicillin N on addition of methionine or L-S-carboxymethylcysteine to suspensions of washed mycelium of *Cephalosporium acremonium*. The same result was obtained by reduction of aeration of such suspensions. This led us to speculate about the possibility that L-S-carboxymethylcysteine or some derivative thereof might interfere with a key enzyme involved in an oxidative process essential for the synthesis of cephalosporin C. L-CMC, because of its thioether group could possibly play the role of a competitive inhibitor for an oxydase system acting on the sulfur atom of an intra-cellular β-lactam containing compound (IV, Fig. 8). Therefore we propose a sulfoxide such as compound V (Fig. 8) as intermediate for cephalosporin C synthesis. This hypothesis is supported by the discovery of chemical conversion from penicillin to cephalosporin *via* sulfoxide formation[24].

Fig. 8. Hypothetical branched pathway for biosynthesis of penicillin N/cephalosporin C/RIT 2214 and tentative explanation for lack of cephalosporin production by *Acremonium chrysogenum* ATCC 20389 in presence of L-S-carboxymethylcysteine.



Traces of an intra- and extra-cellular β-lactam compound totally different from those normally found in *Cephalosporium acremonium* have been detected in cultures of *Acremonium chrysogenum* ATCC 20389 supplemented with L-α-AAA or L-CMC[25]. Most of their bio-activity is directed against Gram-positive microorganisms. They are destroyed by penicillinase treatment and resemble penicillins with hydrophobic side chain in their chromatographic behaviour. The β-lactam compound appearing in the presence of L-CMC persists in the culture broth but the one detected when L-α-AAA was used as precursor remains intra-cellular and disappears just before the onset of cephalosporin C production. It is tempting to consider these substances as intermediates for cephalosporin C biosynthesis. Nevertheless, the identity and exact role of these β-lactam compounds remain speculative.

RIT 2214 and penicillin N have many features in common, but the former can be produced more readily and seems to be more stable than penicillin N. SYLVESTER *et al.*[26] found approximate equal inactivation rates for synnematin B (=penicillin N) and penicillin G at pH 3.0 and 37°C.

The discrepancy between the *in vitro* activity (Table 3) and the *in vivo* activity (Table 4) of RIT 2214 as compared with ampicillin can be explained, at least partially, by the higher plasma concentrations

provoked by subcutaneous injection of the former penicillin[25].

Acknowledgements

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