ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 1973, p. 380-383

Vol. 3, No. 3
Printed in U.S.A.

Bromomonamycins, Unnatural Analogues of the Monamycin Cyclodepsipeptide Antibiotics: Production, Isolation, and Biological Activity

M. J. HALL,[1] B. O. HANDFORD, C. H. HASSALL,[1] D. A. S. PHILLIPS, AND A. V. REES

Department of Chemistry, University College of Swansea, Singleton Park, Swansea SA2 8PP, United Kingdom

Received for publication 2 November 1972

Growth of *Streptomyces jamaicensis* in a low-chloride medium supplemented with NaBr resulted in the biosynthesis of brominated analogues of the natural chlorinated monamycins. Purification of these new compounds was undertaken by Craig countercurrent distribution studies. Diffusion and dilution assays indicated that antibiotic activity of a monamycin was inversely proportional to its unsolvated molecular weight. The bromo analogues were the least active of the series.

The biosynthetic substitution of bromine for chlorine has been achieved in a number of antibiotic substances (3, 6, 8). After the addition of inorganic bromide to a dechlorinated medium, *Streptomyces aureofaciens* produced bromotetracycline (3), and *Penicillium griseofulvum* and *P. nigricans* produced bromogriseofulvin (6). However, formation of the latter could not be induced by using *P. patulum* (7), illustrating that such manipulations were strain or species dependent.

Of the 15 monamycin congeners produced by *S. jamaicensis*, 6 contained chlorine (1). In view of the earlier successes, attempts were made to induce the formation of the bromo analogues in order to compare their antibacterial activity with the natural compounds and produce a single congener suitable for X-ray analysis. Chloromonamycins G_1, G_2, and G_3 were isomeric (mol wt 711) as were H_1 and H_2 (mol wt 725). Chloromonamycin I was a single compound (mol wt 739). The successful incorporation of bromide would produce new compounds having molecular weights of 755/757, 769/771, and 783/785, designated here bromomonamycin 1, 2, and 3, respectively (Table 1).

MATERIALS AND METHODS

The strain of *S. jamaicensis*, the composition of the seed medium, the preparation of the inoculum, and the thin-layer chromatographic system employed were all described previously (4).

[1] Present address: Roche Products Limited, Welwyn Garden City, Hertfordshire, England.

Growth medium. After extensive investigations, a chloride-free medium (CFM) was designed with the following composition: β-alanine, 0.8 g; urea, 0.6 g; glucose, 50 g; $CaCO_3$, 0.5 g; NaH_2PO_4, 23 mg; Na_2HPO_4, 7 mg; $FeSO_4 \cdot 7H_2O$, 7 mg; $ZnSO_4 \cdot 7H_2O$, 7 mg; $MgSO_4 \cdot 7H_2O$, 14 mg; $(NH_4)_6Mo_7O_{24}$, 2 mg; $MnSO_4 \cdot 4H_2O$, 2 mg; $CuSO_4 \cdot 5H_2O$, 2 mg; and distilled water to 1 liter. Bromide was provided by the addition of 175 mg of NaBr per liter. Various vitamins were added at the outset of the work, but their use was discontinued with little effect on yield. All compounds were of analytical grade.

Cultivation. Conditions employed were similar to those used to produce the natural monamycins (4). Erlenmeyer flasks (250-ml, containing 55 ml of medium per flask) were briefly sterilized at 121 C for 15 min and shaken on an orbital incubator (5-cm diameter throw) at 200 rpm and 26 C. Scale-up to a 120-liter fermenter (New Brunswick Fermacell) was achieved by employing a mash volume of 100 liters (26 C, 0.25 vol per vol per min) of air and 150-rpm stirring speed. The course of the fermentation was slower, and maximum titer was not reached until 65 to 70 h compared with the usual 39 to 43 h.

Isolation of bromomonamycin. The monamycin produced during several 100-liter fermentations was extracted into light petroleum (boiling point 40 to 60 C) and concentrated to a light butter-colored oil. The oil was subjected to Craig countercurrent distribution in light petroleum (boiling point 60 to 80 C)–methanol–water (10:10:1) for 100 transfers, which removed the bulk of the contaminating impurities and allowed recovery of crude crystalline monamycin from the first five to six tubes. This material was further subjected to 800 transfers in ethylacetate–cyclo-

TABLE 1. *Accurate mass measurements and molecular formulae of the newly isolated bromomonamycins and their relationship to the chlorinated congeners*

Chloromonamycin	Mol wt	Mol wt of corresponding bromo-analogue	Accurate masses of isolated bromo analogues	Molecular formula
G_1, G_2, G_3	711	755 and 757	755.3218 ± 0.0038	$C_{33}H_{54}O_8N_7{}^{79}Br$
			757.3198 ± 0.0038	$C_{33}H_{54}O_8N_7{}^{81}Br$
H_1, H_2	725	769 and 771	769.3374 ± 0.0038	$C_{34}H_{56}O_8N_7{}^{79}Br$
			771.3354 ± 0.0039	$C_{34}H_{56}O_8N_7{}^{81}Br$
I	739	783 and 785	783.3531 ± 0.0039	$C_{35}H_{58}O_8N_7{}^{79}Br$
			785.3511 ± 0.0039	$C_{35}H_{58}O_8N_7{}^{81}Br$

hexane–methanol–water (41:35:35:10), which separated unhalogenated from halogenated monamycins ((D. A. S. Phillips, Ph.D. thesis, Univ. of Wales, 1969). The countercurrent separations were carried out in a 200-tube Craig machine. The halogenated monamycins peaked at tube number 435, fractions containing only unhalogenated compounds having been removed at intervals to aid the overall separation. The halogenated congeners could not be resolved further by thin-layer chromatography. After 2,400 more transfers in the same system, the halogenated compounds were separated into several overlapping bands. Samples from selected tubes within these bands were analyzed by mass spectrometry (Associated Electrical Industries, MS9) to ascertain which contained the bulk of the brominated compounds. The purest fraction, which was rich in congener 769/771, peaked at tube 1,245; fractions containing the other congeners were removed at intervals to facilitate the overall separation. Tubes with similar contents were pooled, and the solid material was recovered for further purification.

Determination of biological activity. The large plate assay was described previously (4). The turbidimetric assay employed a mannitol broth (MB) with the following composition: yeast extract (Difco), 2.5 g; Peptone P (Oxoid), 5 g; lactose, 2 g; mannitol, 10 g; NaCl, 5 g; Na_2HPO_4, 5 g; tap water to 1 liter. The pH was adjusted to 6.8 with 4 M HCl prior to autoclaving at 121 C for 15 min. Monamycin samples were added in ethyl alcohol, the concentration of which did not exceed 1% (vol/vol) in any single tube. Tubes were inoculated with 50 μliters of log-phase *Staphylococcus aureus* (NCTC 6571) and incubated without stirring at 37 C. The bacteriostatic level was taken as the tube showing no turbidity after 48 h of incubation. The bactericidal level was determined by plating out 0.1-ml samples from each tube onto mannitol agar (MB plus 15 g/liter of agar) and observing for the growth of colonies.

RESULTS AND DISCUSSION

In the tetracycline and griseofulvin fermentations (3, 6), chloride was preferentially incorporated when both chloride and bromide were present in the growth medium. With chloramphenicol, the situation differed, and the bromide competed with the chloride to an extent which depended on the ratio of the two ions in solution (8). The formation of bromomonamycin paralleled the latter rather than the former case, both chloro- and bromomonamycins being coproduced. Such coproduction presented one of the major problems in this study for, although chromatographic methods readily separated the non-halogenated from the halogenated monamycins, they were inadequate for distinguishing between the brominated and the chlorinated congeners. The method adopted eventually to estimate the relative abundance of the various congeners present in a given sample involved measurement of their respective peak intensities in a mass spectrum of the sample. Although, at best, this procedure was only semiquantitative, it was the only convenient method for the purpose available to us at that time. Upon raising the NaBr level from 70 to 150 mg/liter, the peak height ratio, $I_{769} + I_{771}/I_{725}$, rose from 0.88 to 1.7, showing a probable doubling in the proportion of bromomonamycin 2 to chloromonamycins H_1 and H_2. After a series of such studies, an optimum NaBr concentration of 175 mg/liter was adopted for routine use.

By contrast, many experiments were conducted, aimed at reducing the chloride content of the broth, in the hope of eliminating the chloromonamycins. Complex medium "F" used in the routine production of monamycin (4) was dechlorinated either by titration with silver nitrate or by treatment with an ion-exchange resin (Amberlite MB-1). In the latter case, essential cations were restored to the medium in the form of a chloride-free salt solution. The near elimination of chloride by either method caused the formation of chloromonamycins to be almost totally suppressed but, unfortunately, the addition of even high levels of NaBr failed to induce biosynthesis of the brominated congeners.

A similar situation was observed when medium CFM was inoculated with a suspension of mycelial fragments which had been previously washed and suspended in sterile water. Complex seed

medium (1 to 1.5 ml) inoculated directly into medium CFM (4) introduced low concentrations of chloride resulting in the coproduction of bromo- and chloromonamycin. Washing the inoculum to reduce chloride to very low levels inhibited the formation of any halogenated compounds. It was concluded, therefore, that low-chloride levels were essential to initiate or maintain the incorporation of bromide.

The best yields obtained after optimization of medium CFM and the fermentation conditions, and using unwashed inoculum, were about 3 g of total monamycin from a single 100-liter fermentation, of which about 15% represented the bromo compounds. This was considerably below the 10 to 15 g/100 liters obtained with medium "F" in the routine production of monamycin.

The above studies were not successful in completely suppressing formation of the chloro compounds. Craig countercurrent separations did allow isolation of the individual brominated compounds, albeit these were still contaminated with small amounts of certain chlorinated congeners. Accurate mass measurements on each compound (Table 1) confirmed the molecular formulae. The most abundant congener was bromomonamycin 2 (mol wt 769/771). Crude monamycin in the amount of 5.3 g yielded 231 mg of this compound contaminated with 15 to 20% of chloromonamycins H_1, H_2, and I. Confirmation of this residual contamination was also obtained by amino acid analysis of hydrolyzed material (2). Further purification of bromomonamycin 2 is underway to produce crystalline material suitable for X-ray analysis.

The abundance of each of the natural congeners in the crude monamycin mixture varied considerably (2). Only traces of monamycins A and E were present and insufficiently isolated to allow determination of the antibacterial activity. However, the antibacterial activity of the other eight monamycin congeners, including bromomonamycin 2, were compared by the agar-diffusion technique (4). The results (Fig. 1) showed decreasing activity with increasing unsolvated molecular weight. Thus, bromomonamycin 2 was the least active of the series, the unhalogenated analogues were the most active, and the chloromonamycins were intermediate. The general trend of this result was confirmed with some of the more abundant congeners by the use of a turbidimetric assay (Table 2), thereby ruling out the possibility that the results were due to differences in diffusion coefficients in the agar gel. Although the reasons for the above relationship are not apparent, the data imply the

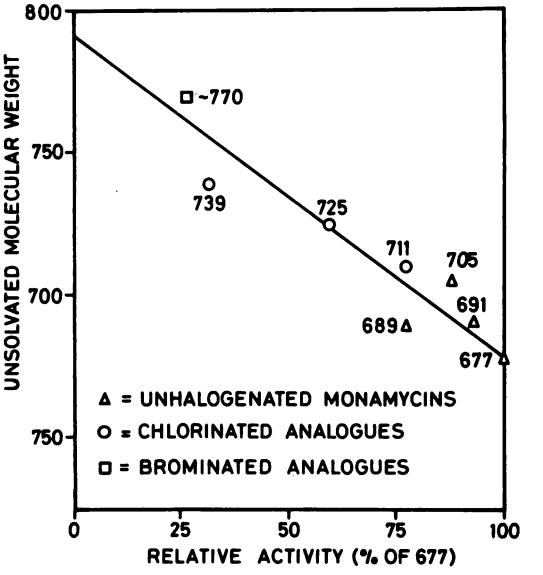

FIG. 1. *Biological activity of eight monamycin congeners relative to monamycin B_1 (mol wt 677) in agar diffusion assay against Staphylococcus aureus.*

TABLE 2. *Bacteriostatic and bactericidal levels of various monamycin congeners in a turbidimetric tube dilution assay against S. aureus NCTC 6571*

Monamycin	Unsolvated mol wt	Bacteriostatic level (μg/ml)		Bactericidal level (μg/ml)
		MIC[a] at 18 h	MIC at 48 h	
$D_1 + D_2$	691	1	3	5
F	705	2	5	>25
$G_1 + G_2 + G_3$	711	1	2	10
$H_1 + H_2$	725	2	10	>25
I	739	4	>25	>25
Bromomonamycin 2	769–771	5	>25	>25

[a] MIC, Minimal inhibitory concentration.

involvement of the halogen and the alkyl substitutents in the mode of action. It is noteworthy that the one consistently anomalous result in the diffusion assays was due to monamycin C (Fig. 1, mol wt 689). This compound is believed to contain the Δ^4- or Δ^5-dehydroderivative of piperazic acid (5) and differs from the other monamycins in possessing a degree of unsaturation. Clarification of these observations may result from the biological and physico-chemical studies at present in progress on the interaction of the monamycins with bacterial cells, erythrocytes, and monovalent cations.

ACKNOWLEDGMENTS

We are indebted to B. Colquhoun and B. Fussell for their technical assistance and to the Science Research Council for the award of a studentship (to A.V.R.).

LITERATURE CITED

1. Bevan, K., J. S. Davies, M. J. Hall, C. H. Hassall, R. B. Morton, D. A. S. Phillips, Y. Ogihara, and W. A. Thomas. 1970. The monamycins, a new family of cyclodepsipeptide antibiotics. Experientia. **26**:122–123.
2. Bevan, K., J. S. Davies, C. H. Hassall, R. B. Morton, and D. A. S. Phillips. 1971. Amino-acids and peptides. X. Characterisation of the monamycins, members of a new family of cyclodepsipeptide antibiotics. J. Chem. Soc. **C**:514–522.
3. Doerschuk, A. P., J. R. D. McCormick, J. J. Goodman, S. A. Szumski, J. A. Growich, P. A. Miller, B. A. Bitler, E. R. Jensen, M. A. Petty, and A. S. Phelps. 1956. The halide metabolism of *Streptomyces aureofaciens* mutants. The biosynthesis of 7-chloro-, 7-chloro^{36}- and bromotetracycline and tetracycline. J. Amer. Chem. Soc. **78**:1508–1509.
4. Hall, M. J., and C. H. Hassall. 1970. Production of the monamycins, novel depsipeptide antibiotics. Appl. Microbiol. **19**:109–112.
5. Hassall, C. H., R. B. Morton, Y. Ogihara, and D. A. S. Phillips. 1971. Amino-acids and peptides. XII. The molecular structure of the monamycins, cyclodepsipeptide antibiotics. J. Chem. Soc. **C**: 526–532.
6. MacMillen, J. 1954. Griseofulvin. IX. Isolation of the bromo-analogue from *Penicillium griseofulvum* and *Penicillium nigricans*. J. Chem. Soc. **II**:2585–2587.
7. Rhodes, A., B. Boothroyd, M. P. McGonagle, and G. A. Somerfield. 1961. Biosynthesis of griseofulvin: the methylated benzophenone intermediates. Biochem. J. **81**:28–37.
8. Smith, C. G., and J. W. Hinman. 1963. Chloramphenicol. Prog. Ind. Microbiol. **4**:137–163.