Phytochemistry, Vol. 23, No. 3, pp. 549–551, 1984.
Printed in Great Britain.

0031-9422/84 $3.00 + 0.00

BIOSYNTHESIS OF CYCLOSPORIN A

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(*Received* 14 *June* 1983)

Key Word Index—*Tolypocladium inflatum*: biosynthesis: cyclic undecapeptide: cyclosporin A; *N*-methylation.

Abstract—Short term feeding of the mould *Tolypocladium inflatum* with ^{14}C-labelled amino acids revealed a selective incorporation of L-leucine, L-valine, glycine and D,L-alanine into cyclosporins A and C. Feeding of L-[*Me*-^{14}C]methionine exclusively labelled the *N*-methyl moieties of the cyclosporins. The distribution of radioactivity from this substrate was directly proportional to the number of the relevant *N*-methyl amino acids in cyclosporin A, indicating a simultaneous methylation of these residues.

INTRODUCTION

Cyclosporin A is a cyclic undecapeptide antibiotic which is active against a narrow spectrum of susceptible fungi [1]. It also exhibits promising immunosuppressive properties [2]. It is produced by the mould *Tolypocladium inflatum* in submerged cultures together with minor amounts of the structurally related cyclosporins B–H [3].

The non-ribosomal biosynthetic pathway, established for other fungal peptides like alamethicin [4] and enniatin B [5], also seems likely for cyclosporin A, since this compound contains the unusual acids α-amino butyric acid, D-alanine and the hitherto unknown (2*S*,3*R*,4*R*,6*E*)-2-methylamino-3-hydroxy-4-methyl-oct-6-enoic acid(C_9-acid). In addition, several peptide bonds are *N*-methylated (Fig. 1).

Abbreviations: *N*-Me-val, *N*-methyl-L-valine; *N*-Me-leu, *N*-methyl-L-leucine; Abu, L-2-aminobutyric acid.

In a recent biosynthetic study, Kobel *et al.* [7] through the use of ^{13}C and ^{3}H NMR spectroscopy after feeding specifically labelled acetate and methionine to *T. inflatum*, concluded that all *N*-methyl groups of cyclosporin A originated from methionine. Furthermore, the unusual C_9-acid was shown to be derived from four acetate units joined by head-to-tail condensation, the *C*-methyl branch originating from methionine. We report in this paper on experiments with ^{14}C-labelled precursors.

RESULTS AND DISCUSSION

T. inflatum was grown in a cornsteep molasses medium, which proved to be very suitable for short-term experiments (10–30 min feeding periods, see Experimental). ^{14}C-Labelled L-valine, L-leucine, D,L-alanine, and glycine, and L-[*Me*-^{14}C]methionine were incorporated into cyclosporin A as the main product with high specific incorporation rates and to a lower extent into cyclosporin

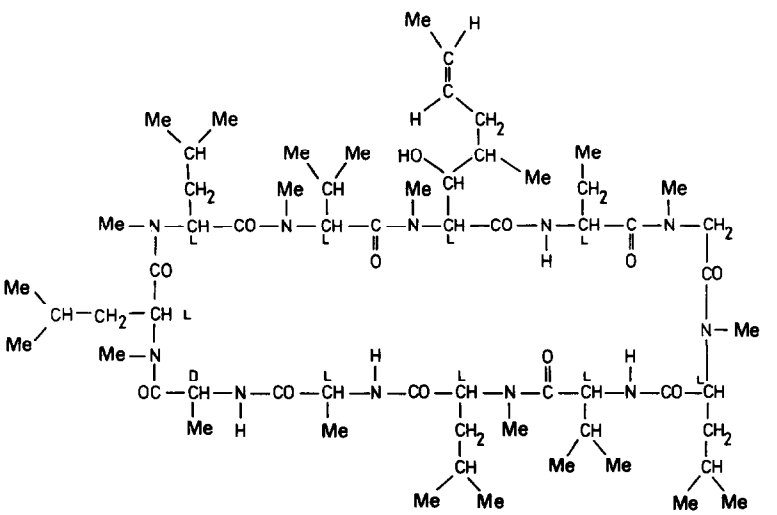

Fig. 1. Structure of cyclosporin A.

C. Radiolabel from each precursor amino acid was found exclusively in the respective constituent amino acid after hydrolysis of cyclosporin A. Cyclosporin C could be selectively labelled by feeding L-[^{14}C] threonine. Feeding of L-[^{14}C]leucine in the presence of excess unlabelled threonine yielded an increased amount of labelled cyclosporin C. Threonine replaces the α-amino butyric acid residue of cyclosporin A in this homologue. This result of our short-term experiments agrees with the previously reported long-term feeding experiments with unlabelled threonine which favoured cyclosporin C over cyclosporin A formation [3]. [^{14}C]Acetate was only weakly incorporated into cyclosporin A and the radioactivity was not enriched in the C_9-acid moiety of the molecule. This result is in contrast to the findings of Kobel *et al.* [7]. The difference might be accounted for by a decreased permeability for acetate of cells grown in our cornsteep-molasses medium. However, in view of the short incubation period, the poor incorporation of acetate could also be interpreted as follows: acetate is not a direct precursor and has to be channeled into the biosynthesis of the C_9-acid. As only the complete C_9-acid can serve as a substrate for the hypothetical cyclosporin synthetase(s), acetate is not incorporated during the short labelling period.

The results shown in Fig. 2 and Table 1 demonstrate that *N*-methylation of valine, leucine, glycine and the C_9-acid occurred simultaneously under our conditions. Thus the radioactivity from L-[*Me*-^{14}C]methionine incorporated into each *N*-methylated amino acid was directly proportional to the number of its residues in the molecule of cyclosporin A. These data confirm the conclusion of Kobel *et al.* [7] that all *N*-methyl groups originate from methionine. According to these authors, the *C*-methyl branch of the C_9-acid is also donated by methionine. However, we observed incorporation of only one methyl equivalent from methionine into the *N*-methyl C_9-acid (Table 1). (Hydrogenation of cyclosporin A yielding a dihydro-C_9-acid moiety was carried out to avoid intramolecular condensation of this unsaturated hydroxy amino acid during acid hydrolysis [8]). Assuming that the methyl equivalent incorporated corresponds to the *N*-methyl group as in the case of the other *N*-methyl amino acids, this result also agrees with the speculation mentioned above, that the complete C_9-acid has to be synthesized before it is accepted by the cyclosporin synthetase(s).

Table 1. Distribution of radioactivity between constituent amino acids of L-[*Me*-^{14}C]methionine-labelled cyclosporin A

Amino acid	cpm
N-Me-dihydro-C_9 acid	527
N-Me-val	532
N-Me-leu	2209
Sarcosine	524
Abu + Ala + Val	34

Radioactivity in the labelled amino acids from the chromatogram shown in Fig. 2 was determined as described in Experimental.

In the case of enniatin biosynthesis, it could be demonstrated that a multifunctional enzyme carries out *N*-methylation of constituent amino acids after their activation as enzyme-bound thioesters [6]. Perhaps a similar mechanism is involved in cyclosporin biosynthesis. This is supported by the failure of ^{14}C-sarcosine (= *N*-methyl glycine) to be incorporated into cyclosporin A (not shown) and the simultaneous *N*-methylation of valine, leucine, glycine and the C_9-acid (Table 1). Based on the data of Kobel *et al.* [7] and of the present study, a tentative sequence of biosynthetic reactions leading to cyclosporin A may be proposed as a working hypothesis: (1) synthesis of all 11 constituent amino acids, (2) activation of all 11 constituent amino acids and (3) *N*-methylation and peptide bond formation.

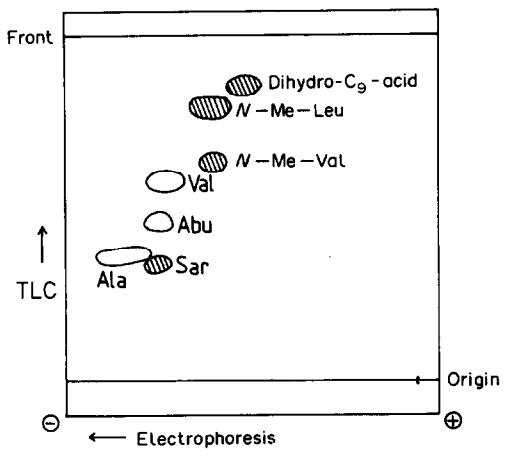

Fig. 2. 'Fingerprint' of L-[Me-^{14}C]methionine labelled cyclosporin A. 2D separation of acid hydrolysates was carried out as described under Experimental. Radioactively labelled compounds are indicated by shaded spots.

EXPERIMENTAL

Tolypocladium inflatum (deposited as *Trichoderma polysporum* DSM 915) was obtained from the Deutsche Sammlung von Mikroorganismen, Göttingen, F.R.G. The medium for submerged cultivation consisted of 1% cornsteep liquor and 3% molasses. Cultures (100 ml medium in 500 ml Erlenmeyer flasks) were inoculated with 10^8 spores. After incubation for 3 days (26°, 115 rpm) on a rotatory shaker, production cultures were inoculated with 5 vol. % of the precultures. After incubation for 30–48 hr, 2 ml of culture were removed, the cells washed with tap water and incubated with 0.5 μCi ^{14}C-labelled amino acid (Amersham, U.K.) for 10 min in the same vol. The mycelial suspension was then extracted with EtOAc and the extractable compounds separated by TLC on silica gel (Kieselgel 60, Merck; EtOAc–MeOH–H_2O, 20:1:1) together with authentic cyclosporin A. Radioactive compounds were detected by autoradiography (Agfa CURIX RP1 film), scraped off the plates and eluted with EtOAc. Acid hydrolysis (6 N HCl, 110°, 22 hr) of cyclosporin was carried out after hydrogenation [8]. 2D-TLC separation of the hydrolysates was carried out according to ref. [9]: Cellulose (Macherey-Nagel, CEL 300); first dimension, electrophoresis at pH 1.9 (HCOOH–AcOH–H_2O, 1:3:20; 1000 V, 1 hr); second dimension, BuOH–AcOH–H_2O (5:1:4). Amino and *N*-methyl

amino acids were detected with ninhydrin reagent. Radioactive amino acids were quantitated after extraction with 50% AcOH by scintillation counting in Bray's soln [10].

Acknowledgements—We thank Dr. H. Kobel, Sandoz AG, Switzerland, for a generous gift of cyclosporin A. This work was supported by the Deutsche Forschungsgemeinschaft (Sfb 9).

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