

BIOTRANSFORMATION OF INDOLE ALKALOID BY TERRESTRIAL STREPTOMYCES GW 14/587 STRAIN INTO A SELECTIVE PHYCOTOXIC AGENT

E.G. Haggag¹ and H. Laatsch²

¹Department of Pharmacognosy, Faculty of Pharmacy, Helwan University at Ain Helwan, Cairo, 11795 Egypt

²Department of Organic Chemistry, George-August-University, Tammann Strasse 2, Gottingen, 27570, Germany

تم فصل مادة مثيل أندول من مستخلص مزرعة بكتريا الإستربتومييس الأرضية فصيلة GW14/587 والتي قد تم تغذيتها بمادة الأندول القلوية بعد يومين من زرعها ثم تركها تطعم لمدة يومين آخرين. تم عمل عدة إختبارات لإختيار أفضل ظروف للتجربة من حيث عدد الأيام اللازمة لتغذية البكتريا لإنتاج أكبر تركيز من المادة الحيوية المنتجة. وقد تم التعرف على التركيب الكيميائي للمادة المنتجة بواسطة الطرق الكروماتوجرافية المختلفة للفصل الكروماتوجرافي المائي ذي الفاعلية والضغط العالي وطيف الكتلة وطيف الرنين المغناطيسي لذرات الهيدروجين والكربون وقد وجد أيضاً أن مستخلص البكتريا المنتج بعد التغذية له فاعلية خاصة ضد سموم الطحالب الدقيقة سكندنييسيمس سيسبيكاتس وهذا ضمن الإختبارات التي أجريت على الكائنات الدقيقة المختلفة المكروبية والفطرية والطحلبية.

Methyl indole was isolated as a metabolite from the culture broth of terrestrial Streptomyces GW 14/587 strain which was fed with indole after two days growth and then inoculated for another two, three and four days to optimize the experiment condition for highest metabolite concentration. The structure of the isolated methyl indole was established using spectral techniques of HPLC-MS, EI-MS, ¹H and ¹³CNMR. Also the bacterial extract showed a high selective phycotoxic activity against Scendensmus subspicatus among the different tested microalgae, yeast, fungi and bacteria.

INTRODUCTION

Microorganisms, in particular bacteria, have been found to have a profound effect on the development of medical sciences. Since the discovery of penicillin in 1929, intensive studies of mainly soil derived bacteria and fungi have shown that microorganisms are a rich source of new biologically active secondary metabolites.¹⁻³ Over the past 60 years between 30,000 and 50,000 natural products have been discovered from microorganisms. More than 10,000 of these compounds were found to be biologically active, of which more than 8,000 are antibiotics and anti-tumor agents. Today, over 100 microbial products continue to be used clinically as antibiotics and anti-tumor agents.⁴

A large variety of indole alkaloids have been isolated from marine organisms,⁵⁻¹⁰ among which is the marine *Streptomyces*.¹¹⁻¹⁴ Terrestrial *Streptomyces* was also investigated for its antibiotic contents.^{15,16} Indole derivatives are common secondary metabolites from microorganisms; more than 1000 derivatives have been reported.⁴

Experiments on feeding bacteria with different compounds and the isolation of their secondary metabolites, preferably those of biological activities, is a new trend.

Here we report on the indole feeding experiment of terrestrial *Streptomyces* GW 14/587 establishing the ultimate inoculation period of the fed media for the bacteria to produce a secondary metabolite which has been isolated, purified and identified. In addition, we report on the antibiotic effect of the extract of

indole fed bacteria on different microorganisms and micro algae.

EXPERIMENTAL

Materials

Terrestrial *Streptomyces* GW 14/587 was obtained from the collection of Alfred-Wegener Institute of Polar and Meeresforschung, Bremerhaven, Germany. *Escherichia coli* (Strain ATCC 25922), *Bacillus subtilis* (Strain Bs 1091-1), *Streptomyces viridochromogenes* (Strain Tu 57), *Staphylococcus aureus* (Strain Sau 1091-5), *Mucor miehei* (Tu 284), *Candida albicans* (HBI 101), *Chlorella vulgaris* (SAG 211-11b), *Chlorella sorokiniana* (SAG 211-8K) and *Scenedesmus subspicatus* (SAG 86-81) were all obtained from stocks maintained at the Department of Organic Chemistry, George-August-University, Göttingen, Germany.

Indole was obtained from Sigma – Aldrich corporation (Germany) and used in a concentration of 400 mg/L culture media using ethanol as a solvent.

Ehrlich's reagent: 0.5 g of *p*-dimethyl amino benzaldehyde (Sigma - Aldrich corporation, Germany) was dissolved in 50 ml methanol and then 2 ml of conc. HCL was added.

Silica gel Alugra SIL G/UV₂₅₄ (Macherey-Nagel GmbH and Co., Duren, Germany) pre-coated aluminum sheets were used for thin layer chromatography. Silica gel Merck 11695 (Sigma-Aldrich Corporation, Germany) was used for preparative TLC.

Equipment

¹H and ¹³C NMR spectra were measured on a varian unity 300 (300.145 MHz) spectrometer in CDCl₃ with TMS as internal standard.

EI-MS was recorded on a varian MAT 731 (70 eV) mass spectrometer, preparative HPLC-MS was performed using an RP18 column (Eurochrome Eurosphere RP 100-C18, 5µm) using a Jasco diode array multiple wave length detector (MD-910) in a scanning range of 195-650 nm, connected to (+ve) ESI Finnigan MAT 95 A mass spectrometer. Retention times were measured on a 4 x 250 nm (Eurochrome RP18 column 60Å, 5µm) with a linear acetonitrile-water azeotrope / water gradient (t = 0: 10%

azeotrope, t = 25 minutes: 100% azeotrope: isocratic for another 50 minutes).

Methods

Preparation of yeast-malt extract medium¹⁷

Malt extract (10 g), yeast extract (4 g) and anhydrous glucose (4 g) were dissolved in distilled water (1 L). Before sterilization, the pH was adjusted to 7.8 by addition of 2 N NaOH.

Fermentation and feeding

Well-grown agar cultures of *Streptomyces* GW 14/587 served to inoculate eight 1-Litre-Erlenmeyer flask each containing 250 ml of yeast malt extract media. The flasks were incubated for two days at 28° with shaking at 95 rpm to grow. The cultures were then fed with 5 ml of prepared indole solution in ethanol in the two following days leaving one group of culture without feeding as a control group. Indole fed cultures were divided into three groups in respect to incubation period; one group was incubated for two days after being fed, the second group was incubated for three days and the third was incubated for four days after being fed; so as to give 4-, 5- and 6-day-old cultures, respectively. All culture flasks were taken out of the shaker and allowed to freeze and lyophilized for 12-24 hours.

Extraction¹¹

The fermentation broth for each group was separately filtered with the aid of celite and the resulting mycelial cake was extracted three times with ethyl acetate each with 250 ml with sonication for 15 minutes. Also the filtrate broth was extracted three times using ethyl acetate. All the ethyl acetate extract from filtrate and mycelia cake, for each group, were combined together, concentrated under reduced pressure and examined by TLC, using dichloromethane/ cyclohexane 8:2 as a solvent system and Ehrlich's reagent as a spray reagent for spot coloring.

Separation

The concentrates of the 4, 5 and 6 days old cultures were separately subjected to preparative thin layer chromatography (PTLC) using cyclohexane / dichloromethane (1:1) as an eluting solvent system. The plates were redeveloped three times to afford a white solid

metabolite (12, 9 and 8 mg of four, five and six day-old culture concentrates, respectively). It gave a dark purplish fluorescence under UV at 254 nm, $R_f = 0.60$ cyclo hexane : dichloro methane; 1:1, an intense blue color when sprayed with Ehrlich's reagent and a reddish brown color spot when dipped in freshly prepared ferric chloride solution.

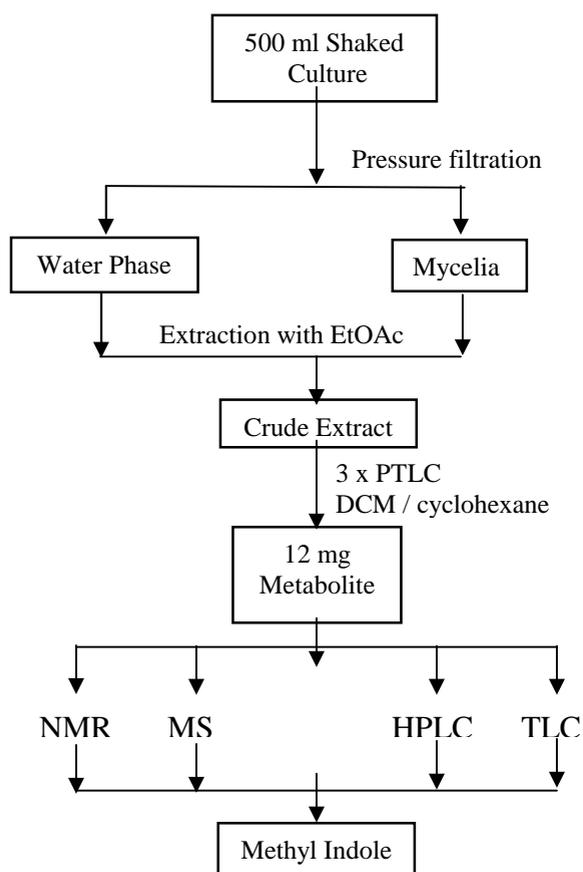


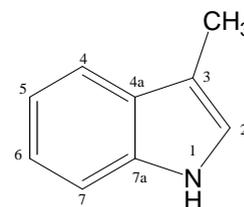
Fig. 1: Work-up of the strain GW 14/587

Antimicrobial activity

Antibacterial, antifungal and antimicrobial (phycotoxic) activities were estimated by the agar diffusion method using paper discs of 8 mm diameter. Each paper disc impregnated with 50 μg of tested culture extract was placed on agar media suspended with test microorganisms. Inhibition zones were observed after incubation at 27° for 24 hours for yeast and 48 hours for fungi, and microalgae and at 37° for 24 hours for bacteria.¹⁸

RESULTS AND DISCUSSION

The structure of the metabolite obtained from four, five and six day-old culture concentrates was established as methyl indole ($\text{C}_9\text{H}_9\text{N}$) using different spectroscopic analyses. EI-MS showed a base peak at m/z 130.1 and a parent peak at 131.1 for M^+ (Figure 2), while HPLC-MS (+ve) ESI showed a base peak at 132.2 for M^{+1} which appeared at R_t 14.45 min (Figure 3). $^1\text{H-NMR}$ showed peaks at δ 8.05 ppm (*s*, br, 1H, H-N), δ 7.65 (*d*, $J=7.5$ Hz, 1H, H-4), δ 7.39 (*d*, $J=7.5$ Hz, 1H, H-7), δ 7.24-7.06 (*m*, 2H, H-5 and H-6), δ 6.95 (*s*, 1H, H-2) and at δ 2.30 (*s*, 3H, CH_3) (Figure 4). $^{13}\text{C-NMR}$ showed nine peaks at δ ppm 136.22 C-7a, 128.22 C-4a, 121.81 C-2, 121.51 C-4, 119.05 C-5, 118.78 ppm C-6, 111.69 C-3, 110.89 C-7 and at δ 9.66 CH_3 (Figure 5). APT- $^{13}\text{C-NMR}$ shows the quaternary carbons C-2, C-4a and C-7a appear down the spectrum base line (Figure 6).



Methyl Indole $\text{C}_9\text{H}_9\text{N}$

This data was confirmed by comparison with data from AntiBase¹⁹ using molecular weight and the substructures determined from the $^1\text{H-NMR}$ data.²⁰ Although methyl indole has been previously synthesized as the known compound Skatole,²¹ this is the first report of its isolation from the *Streptomyces* strain GW 14/587 as a result of biotransformation of the indole base fed into the growth media.

It was reported that²² acriflavine treatment of *Streptomyces* generated a bald mutant that produced two carbazole derivatives, where DNA intercalated the acriflavine dye by mutagenesis. The mechanism of *Streptomyces* strain GW 14/587 to synthesize methyl indole from the indole fed in the media is still under investigation; however mutagenesis could be a possible mechanism.

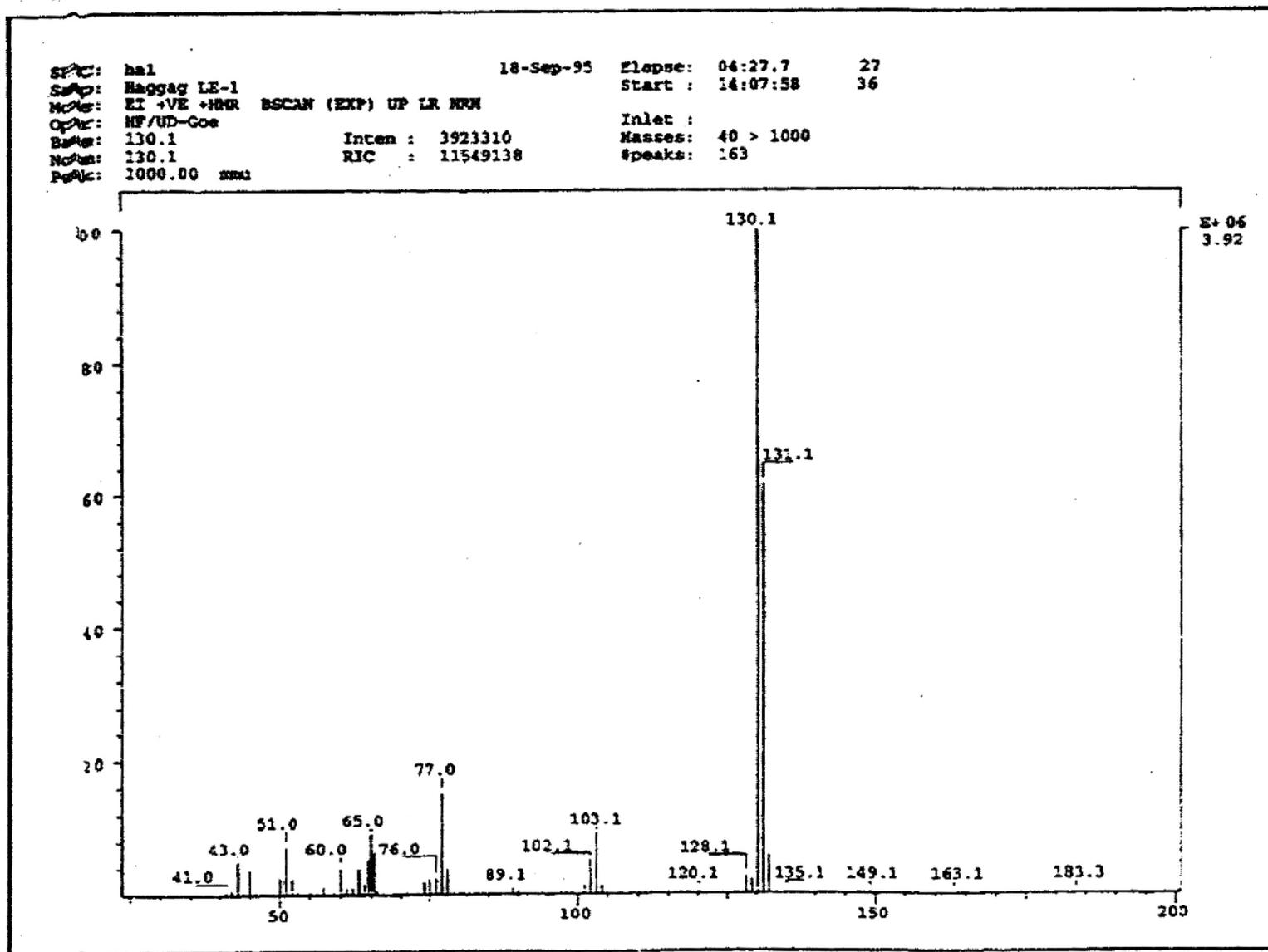


Fig. 2:EI-MS spectrum of methyl indole.

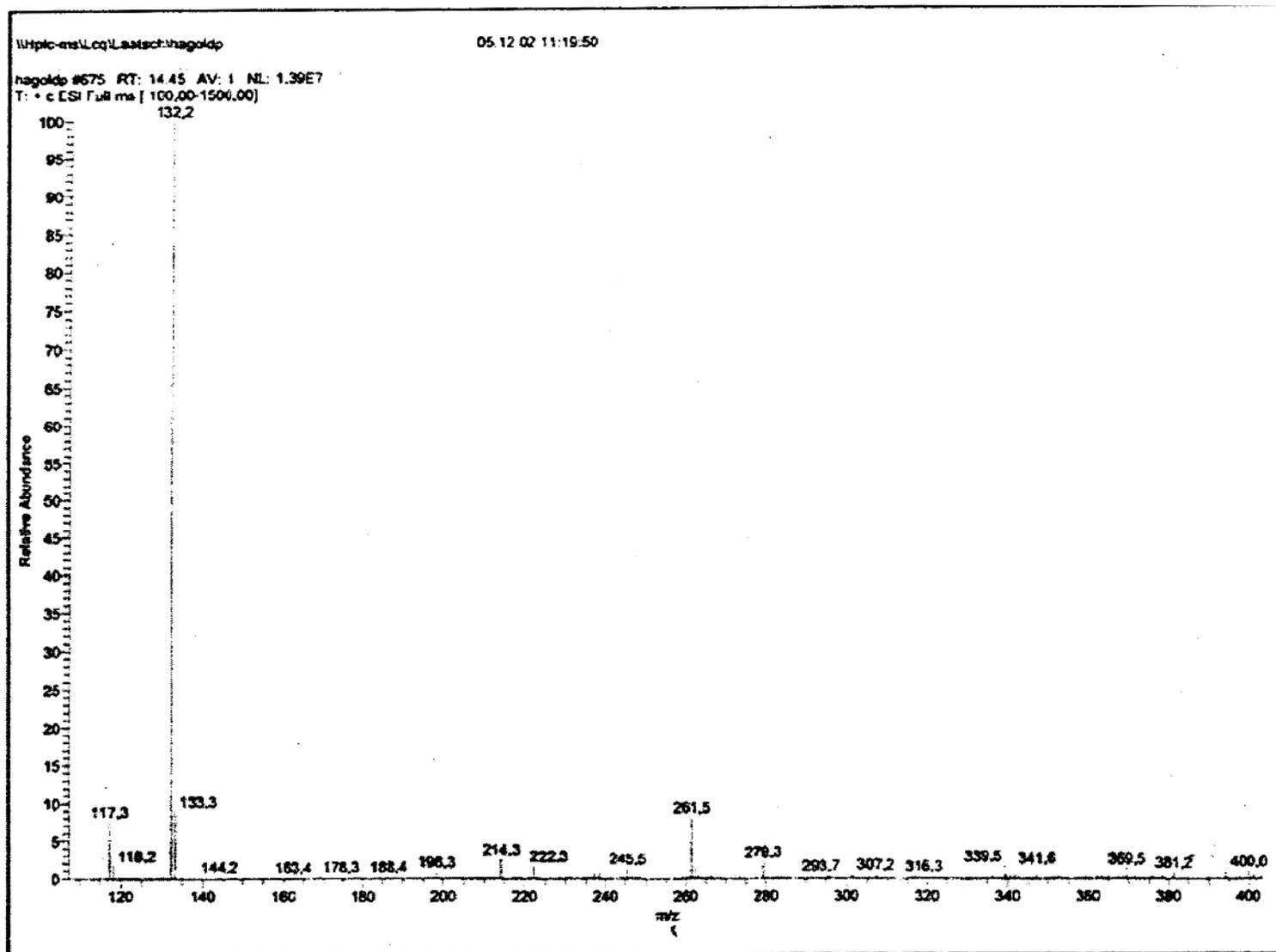


Fig. 3: HPLC-MS (+ve) ESI spectrum of methyl indole.

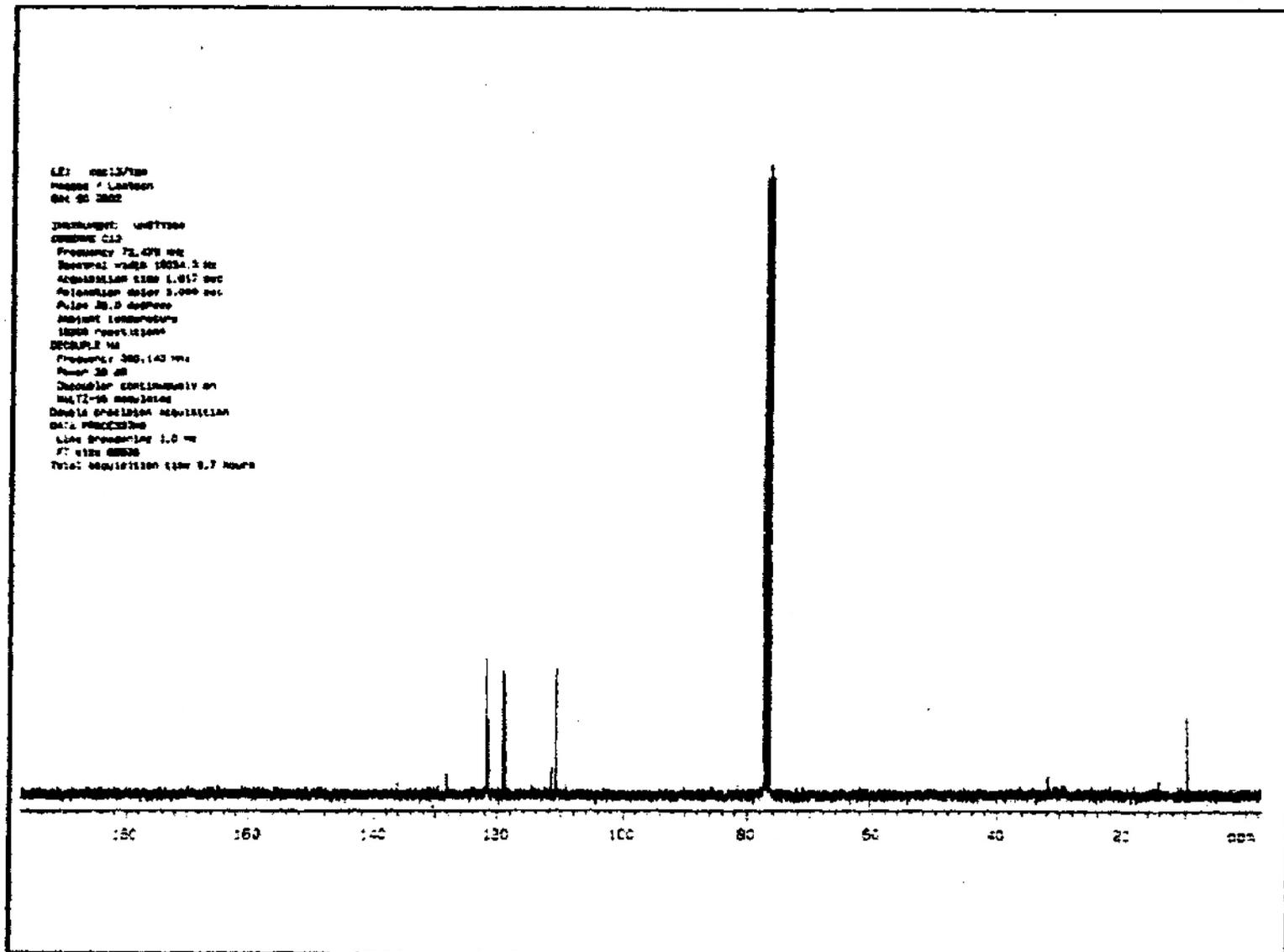


Fig. 5: ^{13}C -NMR spectrum of methyl indole.

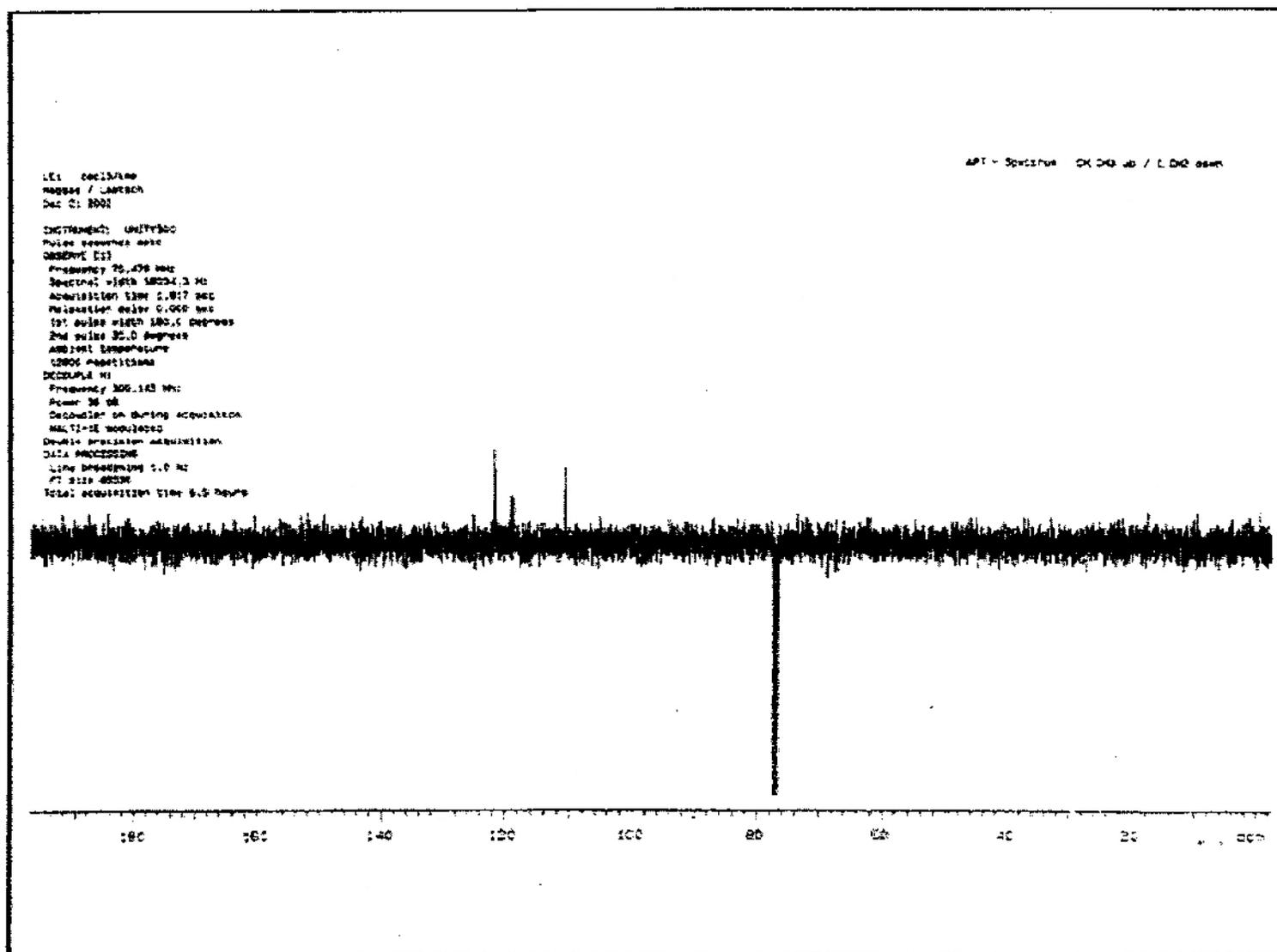


Fig. 6: APT ^{13}C -NMR spectrum of methyl indole.

Table 1: Antimicrobial activities of *Streptomyces* strain GW 14/587 (Parental and indole-fed) culture extracts compared to germanomycine.

	EC	BS	SV	SA	MM	CA	CV	CS	SS
Crude extract of parental culture	0	11	18	0	0	12	15	15	17
Crude extract of indole-fed culture	10.5	11	11	11	0	0	12	12	50
Germanomycine (+ve control)	25	28	22	19	0	0	7	6.5	0

Diameters of inhibition zones in mm.

(EC= *Escherichia coli*; BS= *Bacillus subtilis*; SV= *Streptomyces viridochromogenes*; SA= *Staphylococcus aureus*; MM= *Mucor miehei*; CA= *Candida albicans*; CV= *Chlorella vulgaris*; CS= *Chlorella sorokiniana*; SS= *Scenedesmus subspicatus*)

The high yield of methyl indole produced by the four-days old culture crude extract (12%) was encouraging to test it for antimicrobial activity (Table 1). Feeding *Streptomyces* strain GW 14/587 with indole has changed the antibiotic pattern of the parent strain. As shown in Table 1 the crude extract of the culture fed with indole in its malt media showed in addition to the antibacterial activity of the parent strain against *Bacillus subtilis* and *Streptomyces viridochromogenes*, a moderate activity against *Escherichia coli* and *Staphylococcus aureus*. The parent culture showed moderate activity against the yeast *Candida albicans*, while the fed indole culture extract showed no activity. It was also found that the indole fed culture extract has a remarkably stronger selective antimicrobial activity against *Scenedesmus subspicatus* than the parent crude extract which showed the same moderate phycotoxic activity against the three tested microalgae *Chlorella vulgaris*, *Chlorella sorokiniana* and *Scenedesmus subspicatus*. Both cultures showed no activity against the fungus *Mucor miehei*.

Conclusion

Terrestrial *Streptomyces* strain GW 14/587 showed the ability to synthesize methyl indole from indole fed into its growth media. The biotransformation of indole resulted in a strong active antimicrobial agent against *Scenedesmus subspicatus*.

Acknowledgement

The authors would like to thank Mrs. Fredricke Lissy, Dept. of Organic Chemistry, George-August University, Gottingen, Germany, for her technical assistance.

REFERENCES

- 1- J. d. Faulkner, Nat. Prod. Rep., 18, 1-49 (2001).
- 2- B. S. Davidson, Curr. Opin. Biotech., 6, 284-91 (1995).
- 3- W. Fenical, and P. R. Jensen, Annu. Rev. Microbiol., 48, 559-84 (1994).
- 4- M. Shaaban, R. P. Maskey, I. Wagner-Dobler and H. Laatsch, J. Nat. Prod., 65, 1660-3 (2002).
- 5- J. Kobayashi and M. Ishibashi, In: The Alkaloids, A. Brossi and G.A. Cordell, eds., Academic Press, N.Y., Vol. 41, Chapter 2, 1992, pp. 41-124.
- 6- C. Christophersen, In: The Alkaloids, A. Brossi ed., Academic Press, N.Y., Vol. 24, Chapter 2, 1985, pp. 25-111.
- 7- C. Christophersen, In: Marine Natural Products: Chemical and biological perspective, P. J. Scheuer Ed.; Academic Press, N.Y., Vol. 5, Chapter 5, 1983, pp. 259-85.
- 8- M. Alvarez and M. Salas, Heterocycles, 32, 1391-429 (1991).
- 9- J. Sapi and G. Massiot, In: The Alkaloids, G.A. Cordell, ed., Academic Press, N.Y., Vol. 47, Chapter 3, 1995, pp. 173-226.

- 10- G. W. Gribble, In: Progress in the Chemistry of Organic Natural Products, W. Herz, R.E. Moore, W. Steglich and C. Tamm eds., Springer Wein, N.Y., Vol. 68, Chapter 3, 1996, pp. 141-60.
- 11- M. A. F. Biabani, M. Baake, B. Lovisetto, H. Laatsch, E. Helmke and H. Weyland, *J. Antibiot.*, 51, 333-40 (1998).
- 12- V. J. Mukku, M. Speitling, H. Laatsch and E. Helmke, *J. Nat. Prod.*, 63, 1570-2 (2000).
- 13- R. N. Asolkar, R. P. Maskey, E. Helmke and H. Laatsch, *J. Antibiot.*, 55, 893-8 (2002).
- 14- R. P. Maskey, E. Helmke and H. Laatsch, *J. Antibiot.*, 56, 942-9 (2003).
- 15- M. Abdelfattah, R. P. Maskey, R. N. Asolkar, I. Grun-Wollny and H. Laatsch, *J. Antibiot.*, 56, 539-42 (2003).
- 16- S. Fotso, R. P. Maskey, I. Grun-Wollny, K. P. Schulz, M. Munk and H. Laatsch, *J. Antibiot.*, 56, 931-41 (2003).
- 17- H. Weyland, *Zbl. Bakt. Supp.*, 11, 185-93 (1981).
- 18- B. S. Yun, I.K. Lee, Y. Cho, S. M. Cho I. D. Yoo, *J. Nat. Prod.*, 65, 786-8 (2002).
- 19- H. Laatsch, *AntiBase 2000*, A natural product Database for rapid structure determination, *Chemical Concepts, Weinheim 2000*, see Internet, <http://www.gwdg.de/~ucoc/laatsch/>.
- 20- C. J. Pouchert and J. Behnke, *The Aldrich library of ¹³C and ¹H NMR spectra*, 1993, pp. 121-2.
- 21- Sigma-Aldrich, *Biochemicals and Reagents: for life science research*, 2003, p. 1337.
- 22- H. Grammel, H. Wolf, E. D. Gilles, F. Huth and H. Laatsch, *Z. Naturforsch.*, 53, 325-30 (1998).