Synthesis of a Radiolabeled Enniatin Cyclodepsipeptide [^{3}H-Methyl]JES 1798

U. Pleiss*, A. Turberg†, A. Harder†, M. Londershausen†, P. Jeschke†, G. Boheim‡
*Pharma Product Development, Drug Metabolism and Isotope Chemistry, Bayer AG, 42096 Wuppertal, Germany; †Agricultural Centre, Bayer AG, 40789 Monheim, Germany; ‡Institute for Cell Physiology, Ruhr-University, 44780 Bochum, Germany

Summary

For receptor binding studies and the elucidation of the mode of action of the potent anthelmintic compound JES 1798 a tritium labeled compound at very high specific activity was necessary. Tritium was introduced by methylation of the N-demethyl precursor JES 2314. The identity of [N-methyl-^{3}H]JES 1798 was determined by mass spectrometry. After synthesis and purification, 535 µg [N-methyl-^{3}H]JES 1798 were available at a specific activity of 84 Ci/mmol (3.11 TBq/mmol) as determined by mass spectrometry. The total activity was 80 mCi (2.96 GBq).

Radiolabeled JES 1798 showed an efficient and specific binding to a membrane fraction from *Ascaris suum*. Displacement by unlabeled JES 1798 was half-maximal at about 0.72 ± 0.06 µM. Different known enniatins also competed for the [^{3}H]JES 1798-binding in the *Ascaris suum* membrane preparation. In vitro comparison of JES 1798 with enniatin A, A_1, B and B_1 or beauvericin revealed that enniatin A showed an anthelmintic activity against *Nippostrongylus brasiliensis, Trichinella spiralis* and *Heterakis spumosa* at a concentration of 5 µg/ml, whereas enniatins A_1, B and B_1 had an activity at concentrations between 1 and 100 µg/ml. On the other hand beauvericin and JES 1798 exerted their anthelmintic activities at 100 µg/ml and therefore possess minor anthelmintic potency in vitro as compared to the natural occurring enniatins.

Key words

JES 1798, cyclodepsipeptide, enniatin, anthelmintic, mode of action

CCC 0362–4803/96/070651–09

Received 21 December 1995
Revised 20 February 1996

Introduction

JES 1798 is a non-symmetric cyclohexadepsipeptide consisting of one N-methyl-L-alanine, two N-methyl-L-isoleucines and three D-lactic acid molecules [1,2]. The anthelmintic mode of action of 18-ring-membered cyclodepsipetides, also known as enniatins, is not yet well understood. Therefore, a synthesis of tritium labeled JES 1798 with high specific activity was necessary in order to further elucidate the mode of action of this cyclodepsipeptide in nematodes. An appropriate synthesis was developed.

The present paper describes the synthesis of the [N-methyl-^{3}H]JES 1798. Additionally, preliminary binding studies on membrane fractions from *Ascaris suum* as well as comparative in vitro studies are presented.

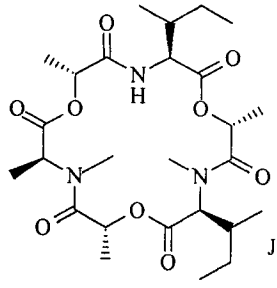
Results

The introduction of a tritiated methyl group into a compound is often a convenient way of labeling. Many drugs, alkaloids and other compounds can be labeled in the methyl group by reaction of suitable intermediates with tritiated methyl iodide [3]. In the case of JES 1798 the experience gained the labeling of the cyclodepsipeptide PF 1022 was used [4]. The precursor JES 2314 reacts only with methyl iodide if the ratio between precursor and methyl iodide is at least 1 : 30 and in the presence of silver oxide.

Caesium carbonate is a better catalyst than silver oxide for the methylation of JES 2314 , but only if the used methyl iodide is absolutely pure and without traces of hydroiodic acid. [^{3}H]methyl iodide is usually prepared from a [^{3}H]methyl ester by cleavage with hydroiodic acid; and any remaining acid then reacts with the excess of silver oxide forming silver iodide. In the case of caesium carbonate the hydroiodic acid does not react and this affects the methylation reaction.

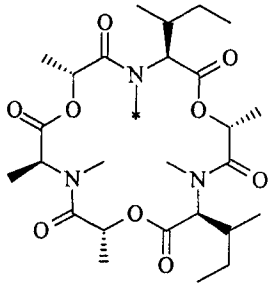
The radiosynthesis was carried out on a 1.2 µmol scale. Approximately 10 Ci (370 GBq) [^{3}H]methyl iodide at a specific activity of 84 Ci/mmol (3.11 TBq/mmol) was condensed with a mixture of silver oxide, JES 2314 and 50 µl dimethyl formamide. After work-up and purification by HPLC 80 mCi (2.96 GBq) [N-methyl-^{3}H]JES 1798 at a specific activity of 84 Ci/mmol (3.11 TBq/mmol) was available and a radiochemical purity of > 94 %. For the planned investigations further purification of the radioactive substance was necessary using HPLC. In this way a radiochemical purity of > 98 % was achieved.

Reaction scheme: Radiosynthesis of [^{3}H]JES 1798



JES 2314

[^{3}H]methyl iodide



[^{3}H]JES 1798

* = [^{3}H]methyl

Figure 1 shows the mass spectrum of JES 1798 and Figure 2 the mass spectrum of [^{3}H]JES 1798. In Figure 1 two strong ions at m/z 556 and 578 can be detected, corresponding to the $[M+H]^+$ and $[M+Na]^+$ ions of the JES 1798. Figure 2 shows ions at m/z 562 and 584 which correspond to the $[M+H]^+$ and $[M+Na]^+$ ions of the triply labeled [N-methyl-^{3}H]JES 1798. The proportions of mono-labeled and di-labeled JES 1798 are only 2 % and 7 %, respectively. Other labeling forms were not detected. Therefore, the specific activity as calculated from the ratio of the peaks of the single, double and triply labeled compounds gave a value of 84 Ci/mmol (3.11 TBq/mmol).

Figure 1: Mass spectrum of JES 1798

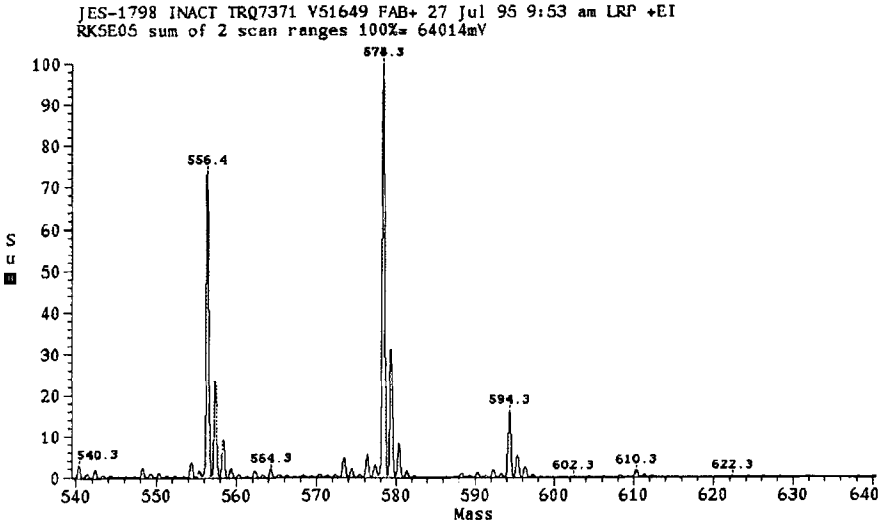
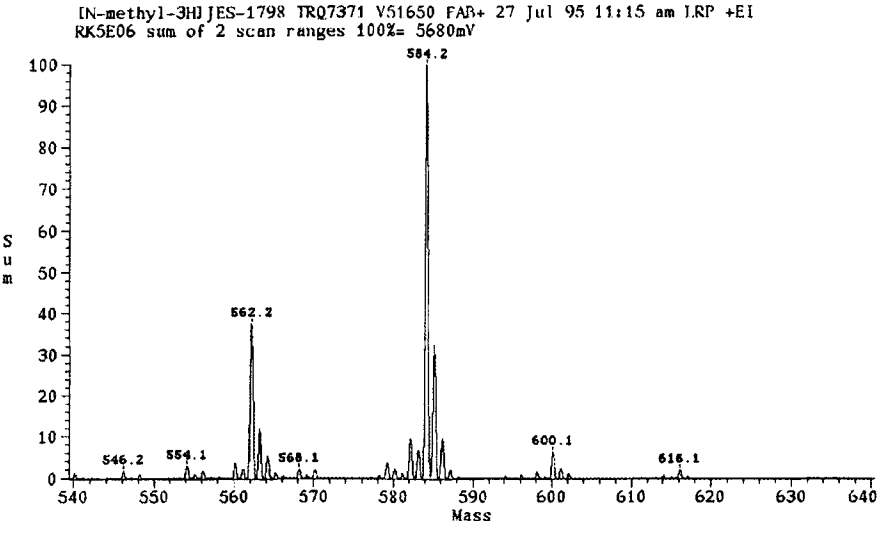


Figure 2: Mass spectrum of [N-methyl-^{3}H]JES 1798



Three different in vitro assays with nematodes living in intestines of rodents (*Heterakis*, *Nippostrongylus*) and muscle tissue (*Trichinella* larvae) were carried out in order to evaluate the anthelmintic effect of JES 1798, beauvericin, enniatin A and A_1, and enniatin B and B_1. Table 1 summarizes the results. JES 1798 exerts similar in vitro anthelmintic effects to beauvericin at concentrations of 100 µg/ml, whereas the naturally occurring enniatins show a somewhat higher activity. The most potent compound is enniatin A, which is fully active at 5 µg/ml (Table 1).

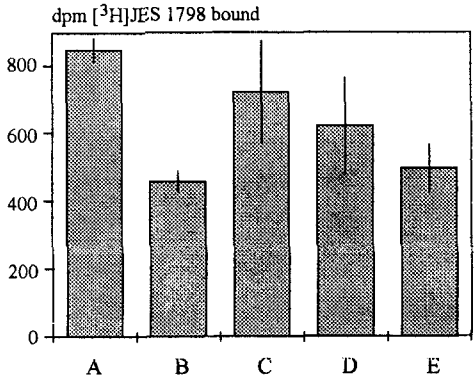
Table 1: Comparison of the in vitro anthelmintic potency of JES 1798 and other enniatins

Compound	Composition	*Heterakis spumosa*		*Nippostrongylus brasiliensis*		*Trichinella spiralis*	
		ppm	efficacy	ppm	efficacy	ppm	efficacy
JES 1798	[-L-MeAla-D-Lac-(-L-MeIle-D-Lac-)$_2$-]	n.d.	n.d.	100	75-99%	100	<50%
Beauvericin	[(-L-MePhe-D-HyIv-)$_3$]	100	<50%	100	75-99%	100	50-75%
Enniatin A	[(-L-MeIle-D-HyIv-)$_3$]	n.d.	n.d.	5	75-99%	5	100%
Enniatin A_1	[-L-MeVal-D-HyIv-(-L-MeIle-D-HyIv-)$_2$-]	1	75-99%	100	<50%	10	100%
Enniatin B	[(-L-MeVal-D-HyIv-)$_3$]	10	<50%	10	50-75%	1	75-99%
Enniatin B_1	[-L-MeIle-D-HyIv-(-L-MeVal-D-HyIv-)$_2$-]	10	75-99%	10	50-75%	1	75-99%

L-MeAla = N-methyl-L-alanine; L-MeIle = N-methyl-L-isoleucine; L-MeVal = N-methyl-L-valine; L-MePhe = N-methyl-L-phenylalanine; D-HyIv = D-2-hydroxyisovaleric acid; D-Lac = D-lactic acid

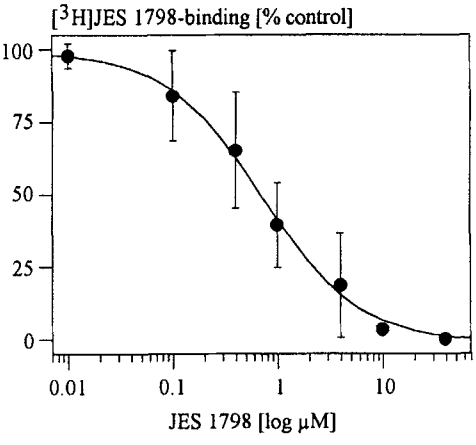
Displacement of 5 nM [^{3}H]JES 1798 binding to membrane fractions from the pig intestinal nematode *Ascaris suum* by 10 µM concentrations of unlabeled enniatins is shown in Figure 3. The best competitor is JES 1798 followed by enniatin B and to a lesser extent enniatin A. Beauvericin did not significantly compete for JES 1798 binding (Figure 3). For JES 1798 an IC_{50} value was determined in five separate experiments and was calculated to be 0.72 ± 0.06 µM indicating high affinity binding of [^{3}H]JES 1798 (Figure 4).

Figure 3: Competition of [^{3}H]JES 1798 binding to an *Ascaris suum* membrane preparation by different enniatins



Membrane preparation: see experimental section. Incubation conditions: 27°C, 0.9 mg protein/ml. Concentration of [^{3}H]JES 1798: 5 nM. Filter assay technique: see experimental section. Competitors added at 10 µM. A = control; B = JES 1798; C = Beauvericin; D = Enniatin A; E = Enniatin B. Line = s.d. (n = 3).

Figure 4: Competition of [^{3}H]JES 1798 binding to an *Ascaris suum* membrane preparation



Membrane preparation: see experimental section. Incubation conditions: 27°C, 1 mg protein/ml. Concentration of [^{3}H]JES 1798: 6 nM. Specific binding is expressed as % of maximal [^{3}H]JES 1798-displacement at 40 µM JES 1798. Filter assay technique: see experimental section. Displacement curve was calculated by a four parameter logistic curve fitting program (Sigma Plot, Jandel Scientific).

Compared to the in vitro activity of the enniatins against nematodes the JES 1798 binding assays show a somewhat different sequence of activity. A possible explanation for these observations might be the structural difference between the enniatins tested with the consequence of altered pharmacological properties like enhanced metabolism or reduced uptake for some enniatins which will both lead to a lower anthelmintic activity. Although less likely, the described differences can also be indicative of several enniatin binding molecules present in the nematode membrane preparation which exert different specificities for the enniatins tested. Since our displacement studies have been performed with one labeled enniatin compound, JES 1798, we were able to determine this particular binding site. The biological assay displays the efficacy of the different enniatins at their specific binding sites respectively, whereas the binding assay determines the sequence of affinity of the various enniatins for the specific e.g. JES 1798 binding site.

Further studies will be carried out to characterize the specific enniatin-binding molecule(s) in nematodes and to compare its properties with a previously described 24-ring membered depsipeptide binding molecule in *Ascaris suum* membrane fractions [4].

Experimental

Synthesis of cyclo(N-[^{3}H]methyl-L-isoleucyl-D-lactyl-N-methyl-L-isoleucyl-D-lactyl-N-methyl-L-alanyl-D-lactyl-), [N-methyl-^{3}H]JES 1798

The tritiation was carried out by Amersham International plc (UK) in accordance with our procedure.

1.2 Mg (2.22 µmol) JES 2314 (precursor substance) and 3.5 mg (15 µmol) silver(I) oxide were weighed into a tapped 5 ml flask. A small stirrer bar was added followed by 50 µl dimethyl formamide, the flask frozen and evacuated. 10 Ci (370 GBq, 118 µmol,) [^{3}H]methyl iodide was distilled in under vacuum and the resulting suspension was stirred at room temperature for 48 hours.

Unreacted [^{3}H]methyl iodide was pumped off and the remainder dissolved / suspended in 0.4 ml acetonitrile and filtered. The solid residue was washed with a further 2 x 0.3 ml acetonitrile, and the combined filtrate and washings were evaporated to dryness. The impure product was dissolved in 0.5 ml ethanol and the solution evaporated to dryness. This procedure was repeated three times. Finally the residue was redissolved in 2 ml acetonitrile and purified by HPLC using the following conditions: column: Chiralcel® OD 250 x 4.6 mm (Daicel); eluent: acetonitrile + water 40 + 60 (v + v); flow rate: 1 ml/minute; UV detection at 228 nm. The specific activity was 84 Ci/mmol (3.11 TBq/mmol) and the total radioactivity 80 mCi (2.96 GBq). These results correspond to a yield of 535 µg (0.95 µmol) [^{3}H]JES 1798 $\hat{=}$ 42.8 % of theory. The radiochemical purity as determined by HPLC was > 94.7 %. This purity was confirmed by TLC on a silica gel plate, eluting with dichloromethane / methanol / 0.88 % ammonia (95 : 5 : 1).

For further investigations the radiochemical purity obtained was insufficient, therefore 5mCi (33 µg) [^{3}H]JES 1798 had to be purified once more under the same conditions mentioned above; the final radiochemical purity was >98.4 %.

2. Membrane preparation

Adult *Ascaris suum* were obtained from a local slaughterhouse. Nematodes in isotonic sodium chloride solution (37°C) were sliced longitudinally and intestines and gonads were removed. Muscles

were scraped from the epidermis and transferred in a glass/teflon potter homogenizer. Ten ml of an ice-cold incubation buffer (67 mM NaCl, 67 mM AcONa, 3 mM KCl, 3 mM $CaCl_2$, 15.7 mM $MgCl_2$, 5% sucrose, 5 mM Tris, pH 7.6 (AcOH) and 0.5 µg/ml leupeptin, 0.7 µg/ml pepstatin, 1 µg/ml aprotinin) were added per two nematodes and the muscle was homogenized by 10 strokes (0°C) followed by centrifugation at 250 g (15 minutes, 4°C). The supernatant was centrifuged at 28000 g (20 minutes, 4°C). Supernatant and lipid layer and a smooth overlaying pellet were removed. The lower pellet was re-suspended in incubation buffer, homogenized in an all-glass potter homogenizer and filtered through 40 nm nylon gauze. The protein concentration was determined according to Bradford [5] and the solution then diluted to about 1 mg/ml.

3. *Binding studies*

Samples were incubated in quadruplicate. Protein concentration and concentration of compounds are given in the legends. Incubations were carried out at 22°C for 30 min. Five ml of ice-cold incubation buffer without protease inhibitors was layered on pre-wetted GF/B glass fibre filters (Whatman) and the incubation was terminated by adding 100 µl of incubated membrane suspension and instantaneous vacuum filtration. Filters were washed three times with 5 ml of incubation buffer without protease inhibitors. Radioactivity retained on the filters was measured by liquid scintillation counting with 5 ml Opti-Fluor (Packard) on a Philips PW 4700 liquid scintillation counter (50% counting efficiency for tritium). Under the conditions described above, non-specific binding to nematode membrane fractions was 60% of total binding.

4. *In vitro evaluation of anthelmintic activity against* Trichinella spiralis *larvae,* Nippostrongylus brasiliensis *and* Heterakis spumosa

Trichinella spiralis larvae were isolated from the skeletal muscles and diaphragm of male SPF/CFW1 mice and stored in 0.9% NaCl, supplemented with 20 µg/ml Clotrimazol®. The incubation of 20 larvae per estimation was performed in 2 ml of a solution, containing 10 g Bacto casitone, 5 g yeast extract, 2.5 g glucose, 0.4 g KH_2PO_4, 0.4 g K_2HPO_4 per 500 ml pH 7.2, supplemented with 10 µg/ml sisomicin and 1 µg/ml Clotrimazol®. 10 mg of the test compound were dissolved in 0.5 ml dimethyl sulfoxide (DMSO) and added to the incubation medium until the final concentrations were 100, 10 or 1 µg/ml. The incubation [6] was carried out over a period of 5 days at 37°C.

Adult *Nippostrongylus brasiliensis* worms were isolated from the small intestine of female Wistar rats and stored in 0.9% NaCl supplemented with 20 µg/ml sisomicin and 2 µg/ml Clotrimazol® [7].

The incubation of each 5 male and female worms was performed in 1.0 ml medium, which was used for estimating of acetylcholine esterase activity [8]. Test compounds were added in the same way as for *Trichinella spiralis* larvae in vitro testing. The evaluation of anthelmintic activity was undertaken after incubation for 6 days at 37°C.

Adult *Heterakis spumosa* worms were isolated from the upper colon of male SPF/CFW1 mice and stored in a salt medium, containing 0.8% NaCl, 0.0175% KCl, 0.0175% $CaCl_2$, 0.01% $MgCl_2$, 5 mM glucose, 1.25 mM K_2HPO_4/KH_2PO_4 pH 7.2, supplemented with 50 µg/l ampicillin and streptomycin and 10 µg/ml Clotrimazol®. The incubation of each 10 female worms was performed in 1.5 ml salt medium. Test compounds were added in the same way as for *T. spiralis* larvae in vitro testing. The valuation of anthelmintic activity was performed after 3 days of incubation at 37°C.

References

1. Jeschke P., Scherkenbeck J., Bonse G., Mencke N., Harder A., Londershausen M., Bischoff E., Müller H., Kurka P. - Ger. Offen DE 4 317 4458 (1992); WO 93/25 543; C. A. 121, 206025 (1994)
2. Jeschke P., Scherkenbeck J., Bonse G., Bischoff E., Mencke N., Harder A., Londershausen M., Müller H. - Ger. Offen DE 4 342 907 (1993); EP 0 658 551; C.A. 123, 340963 (1995)
3. Evans E. A. - Tritium and its compounds (2nd Ed.), Butterworths, London (1974)
4. Pleiss U., Harder A., Turberg A., Londershausen M., Iinuma K., Mencke N., Jeschke P., Bonse G., J. Lab. Comp. Radiopharm., 28: 1 (1996)
5. Bradford M. M. - Anal. Biochem. 72: 248 (1976)
6. Jenkins D. C., Carrington T. S. - Tropenmed. Parasitol. 32: 31 (1981)
7. Jenkins D. C., Carrington T. S. - Vet. Parasitol. 11: 223 (1982)
8. Rapson E. B., Jenkins D. C., Chilwan A. S. - Parasitol. Res. 73: 190 (1987)