Synthesis of a radiolabeled cyclodepsipeptide [^{3}H-methyl]PF1022A

U.Pleiss*, A.Harder†, A. Turberg†, M. Londershausen†,
K. Iinuma‡, N. Mencke†, P. Jeschke†, G. Bonse†

*Pharmaceutical Research and Development, Metabolism and Isotope Chemistry, Bayer AG, 42096 Wuppertal, Germany; †Agricultural Centre, Bayer AG, 40789 Monheim, Germany; ‡Meiji Seika Kaisha, Ltd., 788 Kayama Odawara-Shi, Kanagawa-Ken, 250 Japan

Summary

For receptor binding studies and the elucidation of the mode of action of the potent anthelmintic compound PF1022A a tritium labeled compound with very high specific activity was necessary. Tritium was introduced into the compound by methylation of the [bis-N-demethyl]precursor of PF1022A (PF1022-219). The identity of [bis-N-methyl-^{3}H]PF1022A was determined by LC/MS. After synthesis and purification, 88.9 μg [bis-N-methyl-^{3}H]PF1022A were available showing a specific activity of 162 Ci/mmol (5,99 TBq/mmol) determined by mass spectrometry. The total activity was 15 mCi (555 MBq).

Radiolabeled PF1022A showed an efficient and specific binding to a membrane fraction from *Ascaris suum*. Displacement by unlabeled PF1022A was half-maximal at about 40 nM. At 100-fold higher concentrations the biologically much less effective optical antipodean (PF1022-001) competed for maximal 40% of the [^{3}H]PF1022A-binding in the *Ascaris suum* membrane preparation. In-vitro comparison of PF1022A with its optical antipodean revealed a more than 100-fold higher anthelmintic activity of PF1022A against *Heterakis spumosa*, *Nippostrongylus brasiliensis* and *Trichinella spiralis*.

Key words

PF1022A, [bis-N-methyl-^{3}H]PF1022A, cyclodepsipeptide, anthelmintic, mode of action

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Introduction

Recently, PF1022A (**3**) was isolated from the culture of a parasitic fungal strain on plants [1]. It has been reported that PF1022A exhibits potent anthelmintic activity against parasites of various animals and has no toxic effects on the host animals [1,2]. PF1022A is a symmetric cyclodepsipeptide consisting of 4 N-methyl-L-leucines, 2 β-phenyl-D-lactic acids and 2 D-lactic acids. The mode of action of PF1022A is not yet well understood. Stimulation of the GABAergic and inhibition of the cholinergic system was postulated from electrophysiological data [3]. Therefore, synthesis of tritium labeled PF1022A with very high specific activity was necessary to further elucidate the mode of action of this cyclodepsipeptide in nematodes. An appropriate synthesis was developed.

The present report describes the synthesis of the [bis-N-methyl-^{3}H]PF1022A. Additionally, preliminary binding studies on membrane fractions from *Ascaris suum* as well as comparative in-vitro studies are presented.

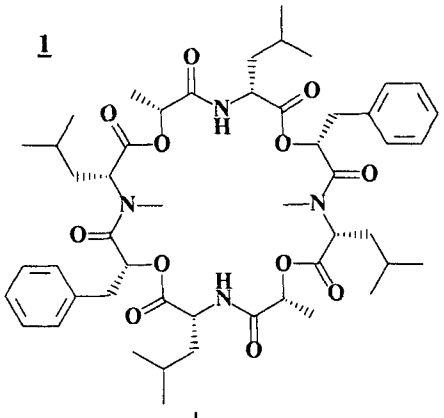
Results and discussion

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 [4]. In the case of PF1022A, it was difficult to find conditions for methylation with methyl iodide. Over 70 different experiments were carried out. Finally, we succeeded in finding the right reaction conditions for the methylation of a demethyl precursor of PF1022A, PF 1022-219 (**1**, see reaction scheme) on a micromol scale.

The precursor PF 1022-219 reacts with methyl iodide only if the ratio between precursor and methyl iodide is at least 1 : 50 and only in the presence of silver oxide . Usual bases like potassium or sodium hydroxide, potassium carbonate, amines or even butyl lithium were not successful. Under these conditions, the reaction rate was better than 90 % (determined by HPLC) relative to the precursor compound PF 1022-219. Preliminary experiments with deuterium revealed a chemical yield of only 30 % after work-up and purification. Apparently, side reactions or adsorption effects on the silver oxide play an important role under the experimental conditions employed.

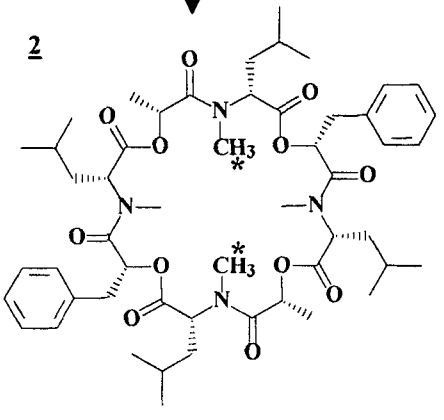
The huge excess of methyl iodide means that for the radiosynthesis at a scale of 1 micromol a starting radioactivity of 4000 mCi (148 GBq) [^{3}H]methyl iodide has to be introduced provided that the specific activity of the [^{3}H]methyl iodide is about 80 Ci/mmol. According to this scale the radiochemical yield can be in the range of 160 mCi (5.92 GBq) (two methyl groups were introduced). But this also means that at the end of the reaction a total activity of 3840 mCi is

Reaction scheme: Radiosynthesis of [³H]PF1022A



PF1022-219
precursor compound

[³H]methyl iodide in toluene
Ag_2O
dimethyl formamide
stirring for 40 hours



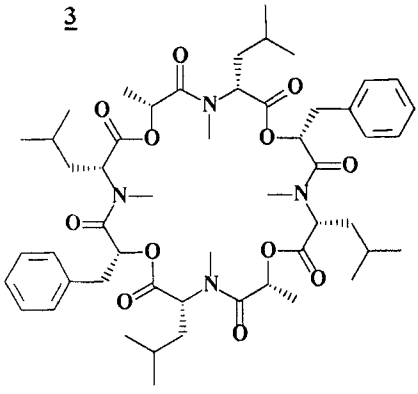
[bis-N-methyl- ³H]PF1022A,
[³H]PF1022A, PLS 0194

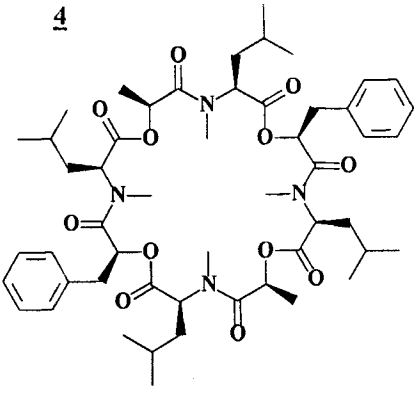
* = labeling position

generated as radioactive waste. The disposal of such an amount of radioactive waste is expensive and difficult. Besides, a manufacturer of radioactive intermediates can certainly re-use the unreacted [³H]methyl iodide for further syntheses. Therefore, we decided to have the radiosynthesis of [³H]PF1022A (2) done by Amersham International plc (UK).

According to our procedure the radiosynthesis was carried out on a 1.2 µmol scale. About 10 Ci (370 GBq) [³H]methyl iodide with a specific activity of 81 Ci/mmol (2.98 TBq/mmol) were condensed onto a mixture of silver oxide and PF 1022-219 dissolved in 50 µl dimethyl formamide. After work-up and purification by HPLC 15 mCi (555 MBq) [bis-N-methyl-³H]PF1022A with a specific activity of 162 Ci/mmol (5.99 TBq/mmol) were delivered by Amersham International plc (UK). The radiochemical purity was > 94 %. The radioactive substance is stored in ethanol at a concentration of 1 mCi/ml (37 MBq/ml) and a temperature of -18 °C. After three months a rate of radiolysis of about 1 % per month was observed.

The [bis-N-methyl-^{3}H]PF1022A was subjected to mass spectrometry which confirmed the chemical identity. The specific activity was calculated from the mass spectrum.





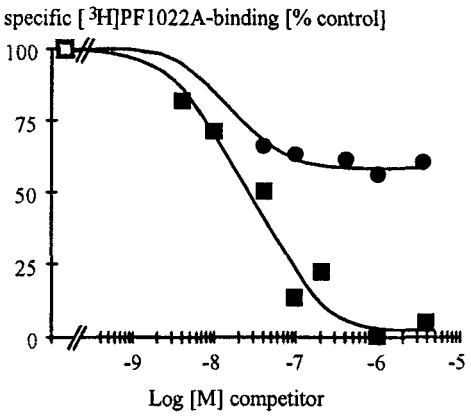
Three different in-vitro assays with nematodes from rodent intestine (*Heterakis*, *Nippostrongylus*) and muscle tissue (*Trichinella* larvae) were carried out for evaluating the anthelmintic effect of PF1022A (3, N-methyl-L-leucin, ß-Phenyl-D-lactic acid, D-lactic acid). In parallel, the optical antipodean - PF1022-001 (4, N-methyl-D-leucin, ß-Phenyl-L-lactic acid, L-lactic acid) - was assayed in the same test systems. Table 1 summarizes the results. PF1022A is about 100-fold more biologically active than its optical antipodean.

Table 1: Comparison of data from biological and binding assays with PF1022A and PF1022-001

compound	Binding assay	In-vitro-assay					
		Heterakis spumosa		*Nippostrongylus brasiliensis*		*Trichinella spiralis*	
	IC_{50}-Value	ppm	efficacy	ppm	efficacy	ppm	efficacy
PF1022A	$4 * 10^{-8}$ M	1	100%	1	75-99%	1	100%
PF1022-001	$> 10^{-5}$ M	100	50-75%	100	40%	100	0%

The stereoselectivity and low effective dose obtained from the biological assays points to a specific binding site for PF1022A in nematodes. The synthesis of tritiated PF1022A enabled us to perform binding studies with membrane fractions from the pig intestinal nematode *Ascaris suum*. First results reveal that binding of 0.14 nM [^{3}H]PF1022A was displaced effectively by PF1022A but merely by PF1022-001. Specific binding corresponds to about 51.2 fmoles PF1022A/mg protein. The IC50 value for PF1022A is given in Table 1. A 1000-fold excess of PF1022A competed for nearly 100% of the specific [^{3}H]PF1022A-binding (Figure 1).

Figure 1: Competition of [³H]PF1022A binding to an *Ascaris suum* membrane preparation



Membrane preparation: see experimental section. Incubation conditions: 0°C, 0.54 mg protein/ml. (□): Concentration of [³H]-PF1022A: 0.14 nM. Specific binding is expressed as % of maximal [³H]PF1022A-displacement at 4 µM PF1022A. Filter assay technique: see experimental section. (■) = PF1022A; (●) = PF1022-001

Investigations with electrophysiological methods on the rat nematode *Angiostrongylus cantonensis* by Terada et al. [3] showed stimulation of the GABAergic action at 10^{-11}-10^{-8} M and an inhibition of the nicotinic action at higher concentrations on the frog rectus muscle. These results also suggest a specific interaction of PF1022A with targets probably located in the neuromuscular system. With tritiated PF1022A we are now able to directly characterize the specific binding site(s) for cyclodepsipeptides.

Experimental

1. Synthesis of [bis-N-methyl-³H]PF1022A

The labeling synthesis was carried out by Amersham International plc. (Cardiff, UK) according to our procedure. 1.4 mg (1.52 µmol) PF1022-219 (precursor substance) and 4.3 mg 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 was frozen and evacuated. 10 Ci (370 GBq, 118 µmol,) [³H]methyl iodide was distilled in under vacuum and the resulting suspension was stirred at room temperature for 40 hours. An intensive yellow colour developed in this time.

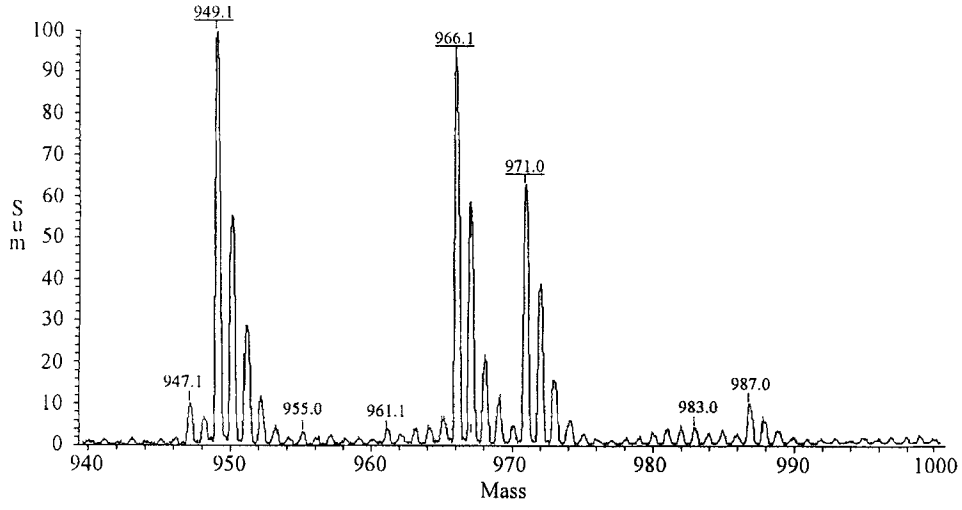
The flask was then evacuated to remove the unreacted [³H]methyl iodide and the dimethyl formamide and the dry residue was dissolved/suspended in 2 ml ethanol and filtered. The solid residue was washed with a further 4 x 2 ml ethanol and 3 x 2 ml acetonitrile, the combined filtrate and washings evaporated to dryness, and 3 x 5 ml ethanol evaporated off to remove labile tritium. The residue was redissolved in 25 ml acetonitrile, assayed and analyzed by TLC. Analysis by TLC on a RP 18 plate, eluting in ethanol : water : acetic acid, showed about 7 % of the activity to correspond to inactive PF1022A marker.

The material from above was evaporated to dryness and redissolved in 1.5 ml acetonitrile/ water 75 + 25 (v + v), giving a yellow solution and an insoluble yellow salt, which was again filtered. The yellow solution was purified by HPLC under the following conditions: column: Ultrasphere® ODS; eluent: acetonitrile + water 75 + 25 (v + v); flow rate: 5 ml/minute; UV detection at 205 nm. The mobile phase containing [^{3}H]PF1022A was fractionated on the basis of the UV and radioactive signal. The solution was evaporated to dryness, and redissolved in 10 ml ethanol. After analysis the solution was diluted with ethanol to a radiochemical concentration of 1 mCi/ml (37 MBq/ml). The total radioactivity was 15 mCi (555 MBq). Because of the high specific activity it was impossible to determine the content of the radioactive substance in the solution by UV. Therefore, the specific activity was determined by LC-MS and turned out to be 162 Ci/mmol (5.99 TBq/mmol). These results correspond to a yield of 88.9 µg (92.5 nmol) [^{3}H]PF1022A which is equivalent to 7.4 % of the theoretically achievable value.

The purity test of [bis-N-methyl-^{3}H]PF1022A, (PLS 0194) was performed under the following conditions: column: Nucleosil® 120, 5 µm, 250 x 4 mm (Muder & Wochele, Berlin, FRG); eluent: acetonitrile + water 75 + 25 (v+v); flow rate: 1.0 ml/minute; UV detection at 250 nm (UV Hewlett-Packard, HP 1050Q, Waldbronn, FRG); radioactivity detector Ramona®4 (Raytest, Straubenhardt, FRG); retention time for [^{3}H]PF1022A, (PLS 0194) = 17.8 minutes. 10 µl of the stock solution were injected corresponding to a radioactivity of 10 µCi (0.37 MBq). Under these conditions the radiochemical purity was > 94 %.

The mass spectrometry of [^{3}H]PF1022A was performed by Amersham International plc.. Figure 2 shows the mass spectrum of PF1022A and Figure 3 the mass spectrum of [^{3}H]PF1022A (PLS 0194).

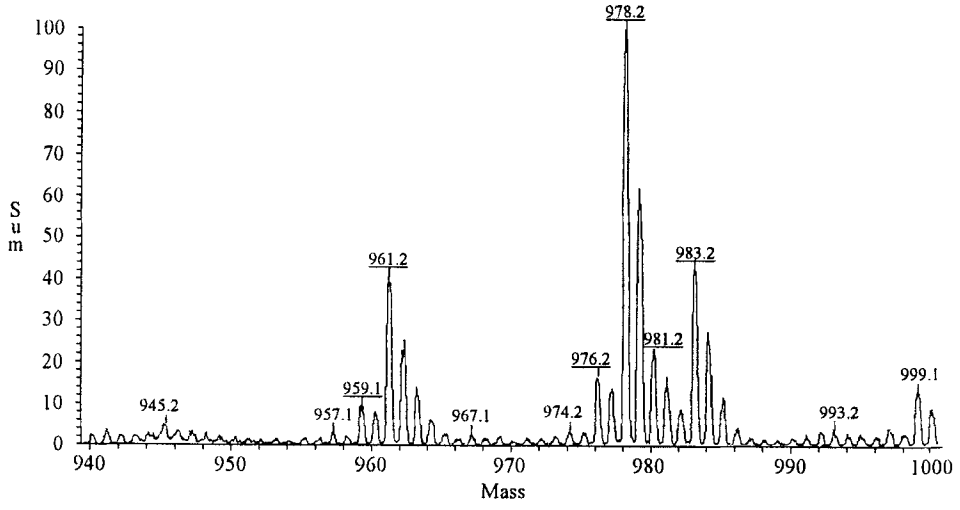
Figure 2: Mass spectrum of PF1022A



PF1022A: sum of two scan ranges 100% = 10354 mV

In Figure 2 three strong peaks at m/z 949, 966 and 971 could be detected which correspond to the $[M+H]^+$, $[M+NH_3]^+$ and $[M+Na]^+$ of the PF1022A. Figure 3 shows peaks at m/z 961, 978 and 983 which correspond to $[M+H]^+$, $[M+NH_3]^+$ and $[M+Na]^+$ of [^{3}H]PF1022A. In addition peaks with m/z 959, 976 and 981 represent the compound which contains only five tritium atoms per molecule. Other labeling states could be not detected. Therefore, the specific activity was calculated from the ratio between the peaks of the fivefold and sixfold labeled compounds to a value of 162 Ci/mmol (5.99 TBq/mmol).

Figure 3: Mass spectrum [^{3}H]PF1022A



[bis-N-methyl-^{3}H]PF1022A: sum of two scan ranges 100% = 7300 mV

2. *Membrane preparation*

Adult *Ascaris suum* were obtained from a local slaughterhouse. Eight nematodes in isotonic sodium chloride solution (37°C) were sliced longitudinally and intestines and gonads were removed. Muscles were scraped from the epidermis and quick-frozen in liquid nitrogen. Muscles were then ground in a liquid nitrogen-cooled mortar and transfered in a glass/teflon potter homogenizer. 10 Ml ice-cold incubation buffer (67 mM NaCl, 67 mM NaAc, 3 mM KCl, 3 mM $CaCl_2$, 15,7 mM $MgCl_2$, 5% sucrose, 5 mM Tris, pH 7.6 (HAc) 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 500 g (15 minutes, 4°C). The supernatant was centrifuged at 28000 g (20 minutes, 4°C). Supernatant and lipid layer were removed, the pellet was resuspended in incubation buffer and re-centrifuged at 28000 g (20 minutes, 4°C). After discarding the supernatant, the pellet was re-suspended in incubation buffer homogenized in an all-glass potter homogenizer. The protein concentration was determined [5] and diluted to about 0.5 mg/ml.

3. *Binding studies*

All glass and plastic ware used in the binding assays were siliconized (Sigmacote, 1 h 70°C). Samples were incubated in quadruplicate. Protein concentration and concentration of compounds are given in the legends. Incubations were carried out at 0°C for 15h and terminated by vacuum filtration of 200 µl membrane suspension through GF/B glass fibre filters (Whatman). Filters were washed twice with 5 ml of incubation buffer without protease inhibitors. Radioactivity retained on the filters was measured by liquid scintillation counting with 8 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 75% of total binding.

4. *PF1022-001*

PF1022-001 (4), the optical antipodean of PF1022A with D-configurated aminoacids and L-configurated lactic acids, used in the binding studies and biological assays was kindly provided by Meiji Seika Kaisha, Ltd..

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

Trichinella spiralis larvae were isolated from skeletal muscles and diaphragm of male SPF/CFW1 mice and stored in 0.9% NaCl, supplemented with 20 µg/ml canesten. 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 sisomycin and 1 µg/ml canesten. 10 mg of the test compound were dissolved in 0.5 ml dimethyl sulfoxide (DMSO) and added to the incubation medium until final concentrations of 100, 10 or 1 µg/ml. After 5 days of incubation at 37°C the experiment was stopped [6].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 sisomycin and 2 µg/ml canesten [7]. The incubation of each 5 male and female worms was performed in 1.0 ml medium, which was taken for the estimation of acetylcholinesterase activity [8]. Test compounds were added in the same way as for *Trichinella spiralis* larvae in vitro testing. The valuation of anthelmintic activity was performed 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 canesten. 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. Takagi M. and Okada T. - Jpn Kokai Tokkyo Koho JP 3,35,796 [91,35,796] (C1C12P21/04), 15 Feb 1991, Appl 90/25, 176, 6 Feb 1990
2. Sasaki T., Takagi M., Yaguchi T., Miyadoh S., Okada T. and Koyama M. - J. Antibiotics 45: 692 (1992)
3. Terada M. - Jpn. J. Parasitol. 41: 108 (1992)
4. Evans E.A. - Tritium and its compounds (2nd Ed.), Butterworths, London (1974)
5. Bradford M.M. - Anal. Biochem. 72: 248 (1976)
6. Jenkins D.C. and Carrington T.S. - Tropenmed. Parasitol. 32: 31 (1981)
7. Jenkins D.C. and Carrington T.S. - Vet. Parasitol. 11: 223 (1982)
8. Rapson E.B., Jenkins D.C. and Chilwan A.S. - Parasitol. Res. 73: 190 (1987)