Journal of Labelled Compounds and Radiopharmaceuticals—Vol. XXXI, No. 4

HPLC ISOTOPE EFFECTS AND MACROCYCLES: THE CASE OF ECHINOCANDIN B

Allen N. Jones*, Robert E. Simpson and Herbert J. Jenkins

Merck, Sharp and Dohme Research Laboratories
P.O. Box 2000, Rahway, NJ 07065

SUMMARY

Echinocandin B was catalytically reduced with hydrogen, deuterium, or tritium. HPLC analysis of the products showed that the labeled analogs exhibited a significant isotope effect relative to the unlabeled parent, with the elution order tritiated < deuterated < hydrogenated. Mass spectral data and specific activity measurements indicated label incorporation exceeded saturation of the double bonds. Proton nmr of the deuterated compound indicated labeling was confined to the aliphatic side chain. A model compound, anilinostearearnide, prepared from the linoleic precursor by reduction with hydrogen or deuterium, incorporated the label only in the aliphatic chain, and also exhibited an isotope effect. This helped confirm that label incorporation in only the fatty acid portion of tetrahydroechinocandin B was sufficient to cause the observed isotope effect.

Key Words: HPLC isotope effect, deuterium, tritium, echinocandin B, anilinostearearnide.

INTRODUCTION

Echinocandin B (**1**) is a macrocyclic peptide possessing antibiotic and antifungal properties[1]. The linoleamide portion readily undergoes catalytic reduction with hydrogen, thereby affording quick and convenient access to labeled analogs. During the course of our analysis of the tritiated compound, we observed that the radioactivity of the tracer was detected prior to the uv absorbance of the reference compound on reversed-phase HPLC (high-performance liquid chromatography). Normally, the converse is observed, since the uv detector precedes the radioactivity detector. In all other respects the tracer and the reference appeared identical. While an isotope effect was suspected, one so significant had not been expected for such a large molecule (mw 1064)[2], and we wanted to verify that: a) no unanticipated structural changes had occurred, b) the label was incorporated only in the aliphatic side-chain, and c) that this labeling was sufficient to cause the observed phenomenon. To this end, both the hydrogen and deuterium analogs were synthesized, analyzed by nmr and mass spectrometry (ms), and their retention times measured by HPLC. In addition, anilinolinoleamide was synthesized as a model compound, reduced with hydrogen or deuterium, and analyzed likewise.

0362–4803/92/040297–07$05.00
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Received 11 December, 1991
Revised 23 January, 1992

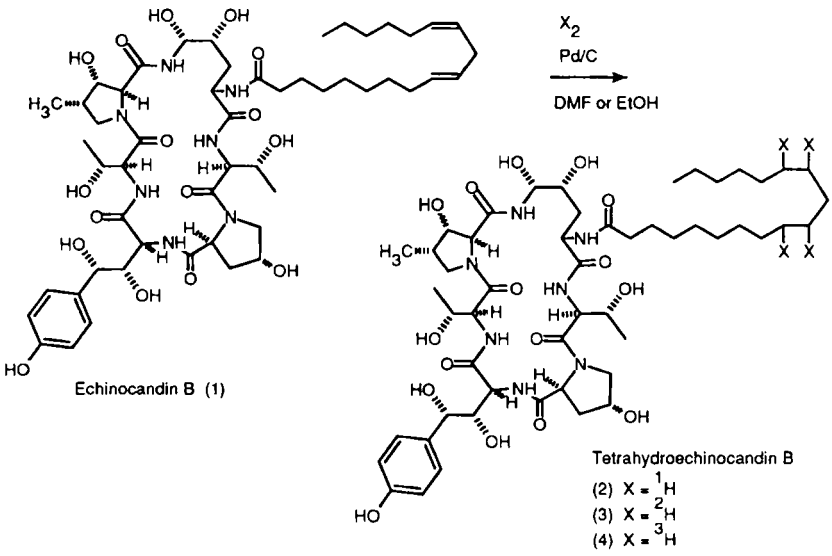
It is well known that substitution of deuterium or tritium for hydrogen on a carbon alpha to an amine results in an increase in the basicity of the amine. Both electronic and steric arguments have been used to explain this observation[3-7]. The consequence on HPLC is an increase in retention time on normal-phase HPLC[8] or a decrease in retention time on reversed-phase HPLC. If the hydrogen isotope is incorporated onto the carbon framework of the molecule, without an adjacent ionizable atom, the rationale for any observed isotope effect is not necessarily clear nor is its occurrence predictable. However, several classes of compounds, when so substituted, have exhibited an isotope effect on HPLC. These include steroids[9,10], vitamin D and its metabolites[11,12], fatty acids[13], and saturated[13] and aromatic hydrocarbons[14,15]. Most of these have contained multiple labeled sites; the last three examples were fully labeled. Thus there was precedence suggesting that our observations with tritiated echinocandin B resulted from a chromatographic isotope effect.

It has been suggested that such an effect arises from differences in interaction between the C-H or C-D bonds and the stationary phase[4,5,7,13-15]. The C-D bonds are shorter, exhibit lower polarizabilities, and have a lower vibrational frequency. Deuterium atoms behave as though they are smaller than hydrogen atoms. Deuterated compounds are less lipophilic than their unlabeled counterparts. The consequence, then, is that the C-D bond would not have as strong an attractive force to the stationary phase as does the C-H bond, and so the deuterated species would elute prior to the hydrogenated species on reversed-phase HPLC. This effect should be further exacerbated with the C-T bond. Obviously, the greater the degree of label incorporation, the greater would be the effect.

RESULTS

Catalytic reduction of echinocandin B with hydrogen readily afforded tetrahydroechinocandin B (scheme 1). The proton nmr spectrum of the reduction product showed a loss of the multiplet centered at 5.35 ppm arising from the olefinic hydrogens of the linoleic acid side chain. The deuterated compound gave an identical spectrum. However, integration of the stearate region for both compounds showed that the latter compound contained approximately eleven fewer hydrogens than the former, indicating that excess deuterium incorporation had occurred. In all other respects, the nmr spectra of starting material and products appeared identical to that which was reported[16].

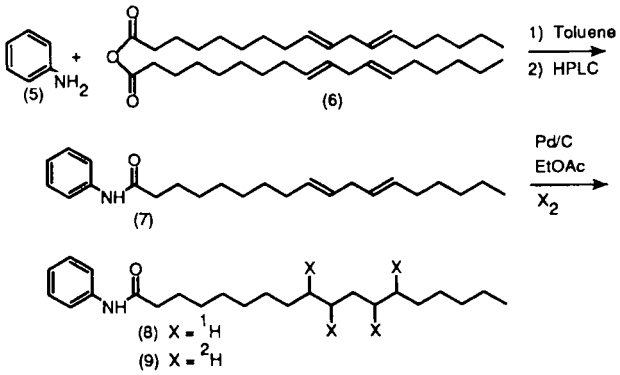
The mass spectrum of the hydrogenated compound gave a parent ion peak at 1087 $(M + Na)^2$. The deuterated analog, however, gave a cluster of parent ions, ranging from 1091 to at least 1101, with the greatest intensity at 1095. This corresponds to the incorporation of four to ten deuterium atoms, with eight predominating. It appears, therefore, that under the conditions employed, allylic labeling occurred, a consequence of double-bond isomerization during the reduction[17]. Hydrogen-deuterium exchange might be occurring as well[18].



SCHEME 1

Tritium incorporation also exceeded saturation of the double bonds, as would be expected. The measured specific activity was 129 Ci/mmol. This is slightly higher than theoretical, which would be 120 Ci/mmol based on incorporation of four tritium atoms. In our experience, the theoretical value is rarely obtained, due to isotopic dilution during reduction.

The aniline derivatives were synthesized as outlined in scheme 2. Nmr, ir, and mass spectral data were consistent with the assigned structures. Comparison of the nmr and ms data for the hydrogenated and deuterated analogs gave results similar to what had been observed for the echinocandins. The stearic acid sidechain of the deuterated compound integrated to roughly eight fewer hydrogens than the hydrogenated compound. The ms gave a parent ion at 360 (M + H) for the hydrogenated compound, while the deuterated species gave a cluster of parent ions ranging from 364 to 370, with 366 predominating. Thus, four to ten deuterium atoms were



SCHEME 2

incorporated, with six being the most favored.

The results of the HPLC analyses are summarized in Table 1. Delta is the difference in retention time between labeled and unlabeled analogs, and is a measure of the degree of separation. Alpha is the ratio of the capacity factors, and is also a measure of the degree of separation. As it increases from a value of 1, the degree of separation increases. I.E. is a measure of the magnitude and direction of the isotope effect. A negative value indicates that the labeled compound elutes prior to the unlabeled one. A value of zero indicates the absence of an isotope effect.

TABLE 1

COMPOUND	RT[a]	SD[b]	K'[c]	DELTA[d]	ALPHA[e]	IE[f]
Tetrahydroechinocandin B						
(2)[g]	17.075	± 0.005	5.098			
(3)[h]	16.809	± 0.026	5.003	0.2660	1.0190	-1.896
(4)[i]	16.749	± 0.004	4.982	0.3260	1.0233	-2.340
Anilinostearеamide						
(8)[g]	16.218	± 0.002	4.7921			
(9)[h]	15.975	± 0.029	4.7055	0.2427	1.0184	-1.842

[a]Retention time (RT) in minutes; each value is the average of three measurements. [b]Standard deviation. [c]Capacity factor; $K' = (RT-t_0)/t_0$; $t_0 = 2.800$ minutes. [d]RT difference in minutes between hydrogenated and deuterated compounds. [e]Ratio of capacity factors; $K'_{unlabeled}/K'_{labeled}$. [f]Isotope effect; $(K'_{labeled}-K'_{unlabeled})*100/K'_{labeled}$[11]. [g]Hydrogenated. [h]Deuterated. [i]Tritiated.

The results show that the labeled compounds consistently elute significantly faster than their unlabeled counterparts. The difference is not great enough to separate the individual components or to resolve labeled compound from unlabeled, but our observations suggest that the possibility exists. The results confirmed our theory that labeling in the aliphatic chain was responsible for the observed isotope effect in tetrahydroechinocandin B. This also lends additional credence to the observation that the whole molecule does not have to be considered when dealing with a chromatographic isotope effect[14].

ACKNOWLEDGEMENTS

We thank Drs. Byron Arison and George Doss for acquiring and interpreting the nmr spectra, and Mr. Jack Smith for acquiring and interpreting the mass spectra.

EXPERIMENTAL

A Beckman System Gold HPLC system consisting of a 126 pump, a 507 autoinjector, and a 166 uv detector was used with Beckman System Gold V5.1 software running on an IBM PS/2-60 computer for data

collection and uv analysis of retention times. An IBM AT computer, running a Berthold LB-506-C-1 radioactivity flowmonitor and version 1.41 of the Berthold software, was used for simultaneous collection of HPLC radioactivity and uv data and subsequent data analysis. Unless noted otherwise, the samples were analyzed on a Whatman ODS-3 column (Partisil-5, 250x4.6 mm) using: A, water/0.1% phosphoric acid; B, acetonitrile/tetrahydrofuran/ phosphoric acid 90:20:0.1; 1 mL/min, 30°C. 55% B was used to analyze the echinocandin derivatives (uv at 210 nm), 80% B was used for the aniline derivatives (uv at 238 nm). Each sample was injected three times, the retention time measured, and the results averaged. The delay time between the detection of the uv peak and the radioactivity peak was measured with tritiated toluene (using 80% organic eluant, uv at 275 nm) and found to be 12 seconds at 1.0 mL/min.

The Echinocandin B reductions were checked for completeness by HPLC using 58:42 acetonitrile/15 mMolar aqueous phosphoric acid, uv at 232 nm, 1 mL/min. Initially, a Zorbax C-8 (250x4.6 mm) column was used (retention time echinocandin B: 5.79 min, retention time tetrahydroechinocandin B: 9.33 min), but changed to a Zorbax ODS 250x4.6 mm, which gave a better separation (retention time echinocandin B: 5.58 min, retention time tetrahydroechinocandin B: 15.26 min). Semi-preparative HPLC was accomplished using either a Spectra-Physics HPLC system for the labeled echinocandins (Zorbax ODS, 250x9.4 mm, 58:42 acetonitrile/15 mMolar aqueous phosphoric acid, 3 mL/min, uv at 232 nm) or an Altex HPLC system for the aniline derivatives (Whatman Partisil 10 ODS-3, gradient from 60/40/0.1 to 80/20/0.1 acetonitrile/water/phosphoric acid in 40 min then to 90/ 10/0.1 at 82 min., 10 mL/min, uv at 254 nm).

^{1}H nuclear magnetic resonance spectra were obtained on CD_3OD (echinocandin derivatives) or $CDCl_3$ (aniline derivatives) solutions with a Varian Unity 400 spectrometer. Infrared spectra was obtained with a Perkin-Elmer 137 ir spectrophotometer. Fast atom bombardment mass spectra (FAB-MS) was obtained with a JEOL mass spectrometer.

EXPERIMENTAL

[^{1}H] Tetrahydroechinocandin B (2)

Echinocandin B (1; 10.0 mg, 9.4 umol) and 10% palladium on carbon (1.0 mg) in deoxygenated ethanol (5.0 mL) was pressurized with hydrogen gas (1 atm) at 25°C and stirred for 2.5 h. The system was purged with nitrogen and the reaction mixture filtered through a 0.5 micron centrifuge filter. The filtrate was diluted to 25 mL with water and adsorbed onto a C-18 solid phase extraction cartridge. The crude compound was eluted with 15 mL of acetonitrile, purified by semi-preparative HPLC, and isolated from the eluant by solid phase extraction to give 2 mg (20%) of tetrahydroechinocandin B.

[^{2}H] Tetrahydroechinocandin B (3)

Echinocandin B (**1**; 10.0 mg, 9.4 umol) and 10% palladium on carbon (1.0 mg) in deoxygenated DMF[19] (2.0 mL, dried over 13X sieves, $(CH_3)_2NH$ free) was pressurized with deuterium gas (1 atm) at 24°C and stirred for 5 h. The system was purged with nitrogen and the reaction mixture filtered through a 0.5 micron centrifuge filter. The filtrate was diluted to 10 mL with water, and purified as for (2) to give 3 mg (30%) of deuterated tetrahydroechinocandin B.

[^{3}H] Tetrahydroechinocandin B (4)

Echinocandin B (**1**; 10.0 mg, 9.4 umol) and 10% palladium on carbon (1.0 mg) in deoxygenated DMF (1.0 mL, dried over 13X sieves, $(CH_3)_2NH$ free) was pressurized with tritium gas (1 atm) at ambient temperature and stirred for 3 h. The system was purged with nitrogen and labile tritium was removed by flushing the reaction mixture several times with dry DMF. The catalyst was then removed by filtration to give a 9.0 mL solution of the crude reduction mixture[20]. A 2.0 mL portion of the DMF solution was diluted to 20 mL with water. The material contained in this solution was adsorbed onto a C-18 solid phase extraction cartridge and eluted with 15 mL acetonitrile to give 60 mCi of crude tracer. Purification was accomplished by repetitive semi-preparative HPLC. Solid phase extraction of the eluant afforded 5.41 mCi of tritiated tetrahydroechinocandin B with a specific activity 129.0 Ci/mmol and having a radiochemical purity of 97.6% by HPLC.

Anilinolinoleamide (7)

Aniline (**5**; 19.1 mg, 0.2 mMole) and linoleic anhydride (**6**; 109.4 mg, 0.2 mMole) were combined in toluene (5 mL) with stirring at room temperature under an atmosphere of nitrogen, then heated at reflux for three hours. After cooling and evaporating the toluene, the product was purified by semi-preparative HPLC to give 60 mg (84%) product.

[^{1}H]Anilinostearamide (8)

Anilinolinoleamide (**7**; 21 mg, 59 uMole) was hydrogenated in ethyl acetate (5 mL) with Pd/C (10 mg) as catalyst (40 psi, 16 hours, r.t.). The catalyst was removed by filtration, and the solvent removed by evaporation at reduced pressure. The dried residue (21 mg, 100%), when analyzed by HPLC, showed no starting material remaining.

[^{2}H]Anilinostearamide (9)

Anilinolinoleamide (**7**; 20 mg, 56 uMole) was deuterated and isolated by the same procedure used for compound **8** to give 20 mg product (100%). No starting material remained by HPLC.

REFERENCES AND NOTES

1. Nyfeler, R.; Keller-Schierlein, W. - Helv. Chim. Acta 57: 2459 (1974).
2. In a large molecule, the presence of naturally occurring isotopes contributes significantly to the molecular weight. In this case, for $C_{52}H_{85}N_7O_{16}$ and using carbon=12, hydrogen = 1, etc., the molecular weight would be 1063. The isotopic abundance, however, contributes one additional mass unit, resulting in the observed molecular weight.
3. Elison, C.; Elliot, H.W.; Look, M.; Rapoport, H. - J. Med. Chem. 6: 237 (1963).
4. Halevi, E.A.; Nussin, M.; Ron, A. - J. Chem. Soc. 866 (1963).
5. Brown, H.C.; McDonald, G.J. - J. Am. Chem. Soc. 88: 2514 (1966).
6. Balaban, A.T.; Stanoiu, I.; Gard, E. - Rev. Roum. Chim 22: 1191 (1977).
7. Heys, J.R. - J. Chromatogr. 407: 34 (1987).
8. Kaspersen, F.M.; van Acquoy, J.; van de Laar, G.L.M.; Wagenaars, G.N.; Funke, C.W. - Recl.: J. R. Neth. Chem. Soc. 103: 32 (1984).
9. Pratt, J.J. - Ann. Clin. Biochem. 23: 251 (1986).
10. Unadkat, J.D.; M. Rowland, M. - J. Chromatogr. 261: 298 (1983).
11. Worth, G.K.; R.W. Retallack, R.W. - Anal. Biochem. 174: 137 (1988).
12. Halloran, B.P.; Bikle, D.D.; J.O., Whitney - J. Chromatogr. 303: 229 (1984).
13. Tanaka, N.; Thornton, E.R. - J. Am. Chem. Soc. 98: 1617 (1976)
14. Baweja, R. - Anal. Chim. Acta 192: 345 (1987).
15. El Tayar, N.; van de Waterbeemd, H.; Gryllaki, M.; Testa, B.; Trager, W.F. - Int. J. Pharm. 19: 271 (1984).
16. Benz, F.; Knusel, F.; Nuesch, J.; Treichler, H.; Voser, W.; Nyfeler, R.; Keller-Schielein, W. - Helv. Chim. Acta 57: 2459 (1974).
17. House, H.O.: - Modern Synthetic Reactions, 2nd ed., W.A. Benjamin, Inc., Menlo Park, CA, 1972, pg 21.
18. Williams, P.G., Morimoto, H., Wemmer, D.E. - J. Am. Chem. Soc. 110: 8038 (1988).
19. DMF (dimethylformamide) was used for the reductions with deuterium and tritium to avoid the isotopic dilution which would occur in protic solvents.
20. Tritium gas reduction carried out at New England Nuclear, Boston, MA.