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RADIO- AND FLUORESCENCE-LABELLING OF THAPSIGARGIN, A SELECTIVE INHIBITOR OF MICROSOMAL CALCIUM-ATPase

ANNETTE ANDERSEN, ANNETTE LAURIDSEN, AND S. BRØGGER CHRISTENSEN*

PharmaBiotec, Department of Organic Chemistry, Royal Danish School of Pharmacy, Universitetsparken 2, DK-2100 Copenhagen Ø, Denmark.

SUMMARY

Debutanoylthapsigargin (*2*) labelled in the skeleton was prepared by stereoselective reduction of the ketone (*3*) obtained by oxidation of debutanoylthapsigargin. Butanoylation of *2* yielded thapsigargin *1*. The use of sodium boro[^{3}H]hydride as a reducing agent afforded labelled debutanoylthapsigargin with a specific activity of 22 Ci/mmol.
A fluorescent analogue of thapsigargin (*4a*) was prepared by allowing *2* to react with N-dansyl-β–alanine. Acetylation of *4a* afforded a trisacetate (*4c*) the missing bioactivity of which allows it to be used as a negative control.

Key words: Thapsigargin, Calcium-ATPase, Deuterium, Tritium, Fluorescence, Dansyl.

INTRODUCTION

The tumour promoting sesquiterpene lactone, thapsigargin (*1*), activates a broad number of cells (1-3). The molecular mechanism behind the activation is a rise in the cytoplasmic calcium concentration induced by an inhibition of microsomal calcium ATPase (4). The specific inhibition of the endoplasmic calcium ATPase has made thapsigargin a key

*To whom correspondence should be addressed.

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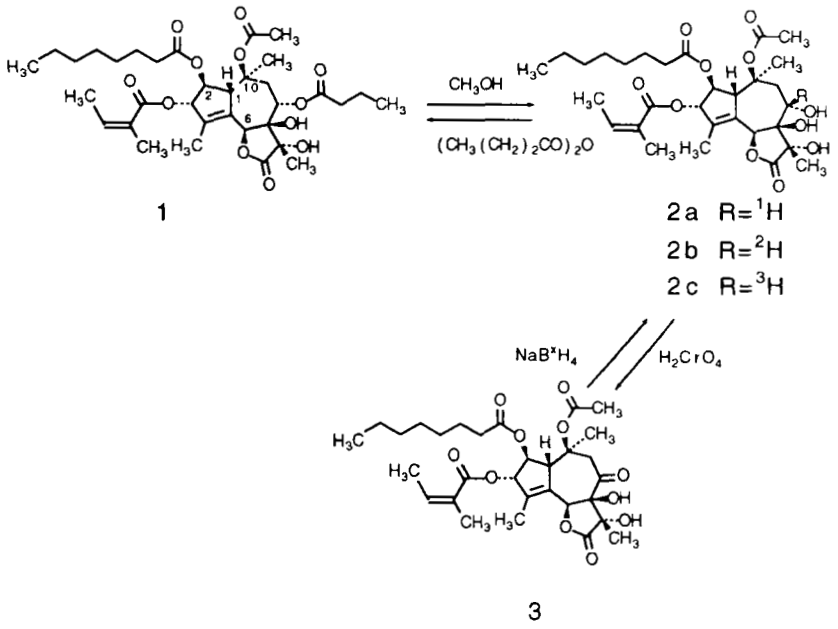
probe for investigation of the calcium homeostasis (5,6) and for studies of the mechanisms behind a number of cellular responses (7,8). In order to get further insight into these processes a method for radiolabelling of thapsigargin has been developed (9). A drawback of this method, however, is, that thapsigargin is labelled in the butanoate group, which, as will be discussed in this paper, is easily lost by solvolysis. Thus, this derivative is unsuited for studies on the metabolism of thapsigargin.

In this paper we describe a methodology for labelling the sesquiterpenoid nucleus. In addition we describe a method for preparing a fluorescent derivative enabling studies on the distribution of the molecule in the cell.

RESULTS AND DISCUSSION

Studies on the stability of solutions of *1* revealed that the molecule is slowly decomposed in a methanolic solution. The degradation product was identified as debutanoylthapsigargin (*2a*). Probably anchimeric assistance from the hydroxy group at C-11 makes the butanoate ester labile. Addition of triethylamine accelerated the solvolysis, whereas *1* was found to be more stable in an acidic solution. Advantage was taken of this observation to develop a method for selective cleavage of the butanoate ester group without opening of the lactone ring. This side reaction has been a previously been a problem (9). The secondary alcohol was oxidized with chromic acid (Jones reagent). The yield of this oxidation, however, is difficult to estimate, since purification by chromatography affords extensive degradation of the ketone (*3*). Reduction of the *3* using sodium borohydride as a reagent affords *2a*. The theoretical possible epimeric alcohol was not detected in the reaction mixture. Use of sodium [^{2}H]borohydride or sodium [^{3}H]borohydride afforded *2b* or *2c*, respectively. The specific activity of *2c* was determined to 22 Ci/mmol. 4-Dimethylaminopyridine catalyzed butanoylation of *2a* afforded *1*.

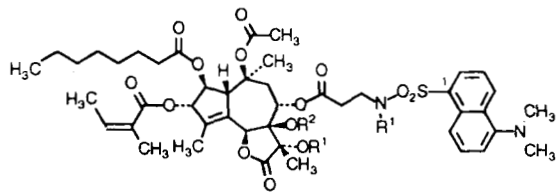


1

2a R=^{1}H
2b R=^{2}H
2c R=^{3}H

3

The easy access to *2a* and the knowledge, that replacement of the butanoyl group with another acyl group only leads to a modification of the biological activity according to the difference in lipophilicity (10), encouraged us to synthesize a fluorescent thapsigargin analogue. If *2a* was allowed to react with N-dansyl-β-alanine in the presence of dicyclohexyl carbodiimide and 4-dimethylaminopyridine the dansylated derivative *4a* was formed in good yield. The lipophilicity of *4a* only makes studies concerning the distribution in the cell valuable if the results can be compared to the distribution of an analogue with no biological activity (a negative control). Structure-activity relationships studies performed on thapsigargin have revealed that the diacetate does not inhibit the calcium ATPase (10). Treatment of *4a*



4a $R^1=R^2=H$
4b $R^1=OCCH_3$ $R^2=H$
4c $R^1=R^2=OCCH_3$

with acetic anhydride in the presence of 4-dimethylaminopyridine at room temperature for three days gave a mixture of the diacetate *4b* and the trisacetate *4c*.

Studies performed on human platelets revealed, that *4a* potently mobilized calcium, *4b* had approximately one tenth the activity of *4a*, and *4c* was inactive (Thastrup, O. and Christensen, S.B. Private communication). A further advantage of *4c* is that the N-acetylation induce a shift of the wavelength of maximum emission enabling a selective determination of each of the compounds in a mixture *e.g.* in the same cell.

EXPERIMENTAL

Column chromatography (cc) was performed over silica gel 60 (0.063-0.200 mm, Merck 7734), tlc was carried out on silica gel 60 F_{254}, Merck). Spots were visualized by UV, spraying with a 0.1 % solution of naphtoresorcinol in M sulfuric acid or by autoradiography using KODAK X-OMAT MA film. HPLC was performed over LiChrosorp RP-18 (Knauer, 8 x 250 mm, 7 μm), eluent: MeOH-H_2O (6:1) added 0.5 % acetic acid, flow 2.5 ml/min, detector: Shimadzu SPD 6A, wavelength: 230 nm. The NMR spectra were recorded on a Bruker AM 250 or AM 500 spectrometer using C^2HCl_3 as solvent. The radioactivity was determined in TRI-CARB liquid scintillation analyzer, model 2000 CA, using OPTIFLOUR Packard as a scintillation fluid. The counting efficiency was determined to 55 % by counting a tritium labelled toluene standard. The fluorescence spectra were recorded on a Kontron Analytical SFM 25 spectrometer in buffered salt solutions containing NaCl (147 mM), KCl (2.7 mM), Sørensen Phosphate buffer pH 7.0 (6.7 mM), bovine serum albumin (1 mg/ml), and glucose (2 mM).

*Debutanoylthapsigargin (*2a*) from thapsigargin (*1*):* Thapsigargin (500 mg, 0.77 mmol) was dissolved in MeOH (50 ml) and triethylamine (2.5 ml) was added. The solution was left for 7 h at room temperature. The solvent was evaporated *in vacuo* to give a gum, from which *2a* (420 mg, 94%) identical to an authentic sample (11) was isolated by cc using toluene-EtOAc (3:2) as eluent. The retention time was by HPLC determined to 9.5 min.

Oxidation of 2a *to give the ketone* 3: Debutanoylthapsigargin (*2a*) (21 mg, 36.2 μmol) was dissolved in acetone (8 ml) and Jones reagent (12) (40 μl) was added and the mixture was left for 40 min. The reaction mixture was filtered and the solvent was evaporated *in vacuo* to give a gum (22 mg). Purification by HPLC [LiChrosorp RP-18, 5 μm (Knauer), MeOH-water (4:1) added 0.5% acetic acid, retention time 10.2 min] led to extensive degradation of *3*, however a small amount was obtained in a pure state. ^{1}H NMR data: δ 5.71 (1H, br.s, H-3), 5.58 (1H, br.s., H-6), 5.45 (1H, t, *J* 4 Hz, H-2), 4.33 (1H, br.s, H-1), 4.09 (1H, d, *J* 12 Hz, H-9), 2.87 (1H, d, *J* 12 Hz, H-9'), 1.92 (3H, br.s, H-15), 1.35 (6H, s, H-13 and H-14); the signals of the acyl moieties were found as previously reported (10).

Reduction of 3 *with* $NaBH_4$ *to give debutanoylthapsigargin* (2a)*:* To a solution of crude *3* (18.5 mg) in isopropanol (9 ml) was added $NaBH_4$ (6.5 mg, 17.2 μmol) and the mixture was left for 2 min at -18°C. After addition of acetone (24 ml) hydrochloric acid (0.8 M, 24 ml) was added to the mixture. The mixture was concentrated to 24 ml *in vacuo* and the residue was extracted with three portions of EtOAc (20 ml). The combined EtOAc-phases were concentrated *in vacuo* to give a slightly yellow gum, which was purified by cc [toluene-EtOAc (4:1) added 1% of acetic acid] to give *2a* (4.5 mg, 7.8 μmol) identical to an authentic sample of *2a* (11).

Reduction of 3 *with* NaB^2H_4 *to give deuterated debutanoylthapsigargin* (2b)*:* The ketone *3* was reduced with NaB^2H_4 following the same procedure as was used for reduction of *3* with $NaBH_4$ using the crude oxidation product containing *3* (22 mg) as starting material and NaB^2H_4 (20 mg, 47 μmol) to give *2b* (4.2 mg, 7.2 μmol). The product cochromatographed with *2a* by HPLC. The ^{1}H NMR spectrum was identical with that of *2a* except for the diminished signal of H-8 (integral ≈ 0.2H corresponding to a deuteration of 80%) and the appearance of the two 9-protons, which both appear as doublets (*J* 15 Hz). A molecular ion at *z/e* 581.2 was observed in the negative FAB-mass spectrum.

Reduction of 3 *with* NaB^3H_4 *to give tritiated debutanoylthapsigargin* (2c)*:* Because of missing access to equipment suitable for handling radioactive gases formed during the

reaction the reduction of the ketone *3* was performed by Amersham, England, according to the protocol described above for the reduction of *3* with $NaBH_4$ using 20 mg of crude *3* as starting material. The working up of the crude reaction mixture and the determination of specific activity and radiochemical purity were done by the authors. The product was purified to give 2*c* (0.55 mg, 0.94 μmol), the radiochemical purity was by tlc [isopropanol-toluene (1:50)] determined to 90% and the specific activity to 22 Ci/mmol. The product co-chromatographed with *2a* by HPLC.

Butanoylation of 2b *to give deuterated thapsigargin*: To a solution of butanoic anhydride (8 μl, 50 μmol) and 4-dimethylaminopyridine (50 mg, 0.4 mmol) in CH_2Cl_2 was added *2b* (6.1 mg, 11 μmol) and the solution was left for 1 h at room temperature. To the solution was added hydrochloric acid (4 M, 5 ml) and the organic phase was isolated and concentrated *in vacuo* to give a gum from which thapsigargin (3.2 mg, 46%) deuterium labelled at C-8 was isolated by cc [toluene-EtOAc (3:1)]. The ^1H NMR spectrum was superimposable with that of *1* (11) except for the diminished signal of H-8 (integral ≈ 0.2 corresponding to a deuteration of 80%) and the appearance of the signals belonging to the two 9 protons, which both appeared as doublets (*J* 12 Hz).

Acylation of debutanoylthapsigargin (2a) *with N-dansyl-β-alanine to give the dansyl derivate* (4a): To a solution of debutanoylthapsigargin (*2a*) (200 mg, 0.34 mmol) in CH_2Cl_2 (5 ml), was added 4-dimethylaminopyridine (42 mg, 0.34 mmol) and N-dansyl-β-alanine (13) (220 mg, 0.68 mmol). The mixture was stirred at 0^oC for 5 min and then added 1,3-dicyclohexylcarbodiimide (82.5 mg, 0.40 mmol). The reaction mixture was stirred for 6 h at 0^oC and for 16 h at 5^oC (14). The dicyclohexylurea precipitate was removed by filtration and the solvent was evaporated *in vacuo* to give a gum, which was dissolved in diethyl ether (15 ml). The organic phase was washed with M HCl and M Na_2CO_3, dried, and concentrated *in vacuo*. The residue was purified by cc [toluene-EtOAc (5:1)] to give the starting material *2a* (18.5 mg, 9.3 %), and *4a* (156,9 mg, 52.2%). ^1H NMR data of *4a*: *The sesquiterpene moiety*: δ 5.67 (1H, br.s, H-3), 5.58 (2H, m, H-6 and H-8), 5.45 (1H, t, *J* 3.4 Hz, H-2), 4.17 (1H, br.s,

H-1), 2.90 (1H, m, H-9), 2.37 (1H, dd *J* 3.9 and *J* 14.8, H-9'), 1.81 (3H, br.s, H-15), 1.49 (3H, s, H-14), 1.31 (3H, s, H-13), *the dansyl moiety*: δ 8.53 (1H, br.d, *J* 8.5 Hz, H-2), 8.21 (2H, m, H-8 and H-4), 7.58 (1H, dd, *J* 7.6 Hz and *J* 8.6 Hz, H-7), 7.51 (1 H, dd, *J* 7.3 Hz and *J* 8.5 Hz, H-3), 7.19 (1H, br.d, *J* 7.6 Hz, H-6), 2.88 (6H, s, $N(CH_3)_2$), *the* β-*alanine moiety*: δ 5.81 (1H, br.t, *J* 5.2 Hz, NH), 3.18 (2H, br.q, *J* 5.2, H-β), 2.43 (2H, br.t, *J* 5.2, H-α); the signals of the acyl moieties were found as previosly reported (10). Fluorescence data of *4a* (λ_{exc} = 352 nm) [emission maximum (intensity)]: 712 nm (35) and 490 nm (100).

Acetylation of the dansyl derivate (4a) *to give* 4a *and* 4b*:* To a solution of *4a* (20 mg, 22.6 μmol) in CH_2Cl_2 (1.5 ml) was added 4-dimethylaminopyridine (10 mg, 0.41 mmol) and acetic anhydride (500 μl, 5.30 mmol). The solution was left for 3 days at room temperature, and then added diethyl ether (10 ml). The organic phase was washed with M HCl and M Na_2CO_3, dried and concentrated *in vacuo*. The residue was purified by cc [toluene-EtOAc (3:2)] to give *4b* (4 mg, 18.2%) and *4c* (9.5 mg, 41.6%). The ^1H NMR spectrum of *4b* was identical with that of *4a* except for the following protons: *The sesquiterpene moiety*: δ 5.49 (1 H, t, *J* 3.8 Hz, H-8), 4.30 (1 H, br.s, H-1), 2.09 (3H ,s, O-11 acetyl), *the dansyl moiety*: 8.12 (1H, dd , *J* 7.1 Hz and *J* 1 Hz, H-4), 8.00 (1H, br.d, *J* 8.9 Hz, H-8), *the* β-*alanine moiety*: δ 4.14 (2H, m, H-β), 2.78 (2H, m, H-α), 2.37 (3H, s, N acetyl), 2.09 (3H, s, O-11 acetyl). Flourescence spectrum of *4b* (λ_{exc} = 285 nm) [emission maximum (intensity)]: 685 nm (100) and 590 nm (34). The ^1H NMR spectrum of *4c* was identical with that of *4b* execept for the following protons: *the sesquiterpene moiety*: δ 6.50 (1H, br.t, *J* 3.0 Hz, H-8), 5.77 (1H, br.s, H-6), 2.26 (3H, s, O-7 acetyl). Fluorescence spectrum of *4c* (λ_{exc} = 285 nm) [emission maximum (intensity)]: 685 nm (100) and 580 nm (67).

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