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UNSATURATED EPOXY-C-GLYCOSIDES. A NEW CLASS OF ANTITUMOR COMPOUNDS WITH DNA CLEAVAGE PROPERTIES.

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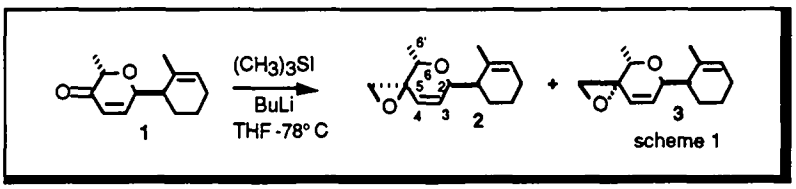
Abstract. α,β–Unsaturated-epoxy-C-glycosides were synthesized by the condensation of 4-keto unsaturated C-glycosides with dimethyl sulfonium methylide. These compounds were tested for cytotoxic activity against Raji and LFCl2a cells. Simple C-glycosides were found unreactive. However, the derivatives of fatty acids have shown a cytotoxic activity comparable to those of the most potent keto C-glycosides. An apoptosis study carried out with these compounds led to the conclusions that epoxy-C-glycosides possess DNA cleavage properties.

The discovery of the antitumoral activity of keto-C-glycosides[1-4] led us to develop a program of analogues with the objective to prepare a second generation of C-glycosides directed against different biological targets. Recent studies from these laboratories[2,4-6] have shown the possibility of a correlation between cellular target (DNA, proteins) and the structure of the fatty acid in fatty acid-drug conjugates. This hypothesis was highlighted by differences between the cytotoxicity of arachidonate and docosahexaenoate derivatives. Thus with DNA alkylating molecules (chlorambucil, 5-deoxyfluorouridine) docosahexaenoate conjugates were the most actives. On the other hand with keto-C-glycosides that were expected to react with the SH group of proteins,[7, 8] arachidonic-drugs conjugate were the most potent.[2]

An answer to these questions may be addressed by the synthesis of C-glycosides directed against DNA and possessing structures as close as possible to keto-glycosides. With the above considerations in mind we selected α-ß unsaturated epoxides as models for this study. Vinyl epoxide has been found recently in new antitumor antibiotics like kapurimycin[9] and it has been demonstrated that this molecule alkylates DNA. Moreover recent studies have reported that sulfuryl radicals react with vinyl epoxide to generate alkyloxy radicals which cleave DNA in vitro.[10] In this letter we report the synthesis and the antitumor properties of α,ß-unsaturated-epoxy-C-glycosides



The general synthesis was carried out by the reaction of α,β-keto unsaturated-C-glycosides with dimethylsulfonium methylide[11] ($(CH_3)_3SI$, BuLi, THF -78°C) (Scheme I). The results obtained are summarized in Table I. The condensation led to the corresponding epoxides with overall yields ranging from 40 to 63%. Epoxidation of methylcyclohexene derivatives led to better results than the methylene cyclohexene-C-glycosides. On the other hand it should be noted that the epoxidation of the hydroxy ketone **4** was quite highly stereoselective affording **5** and **6** in a 94:6 ratio.

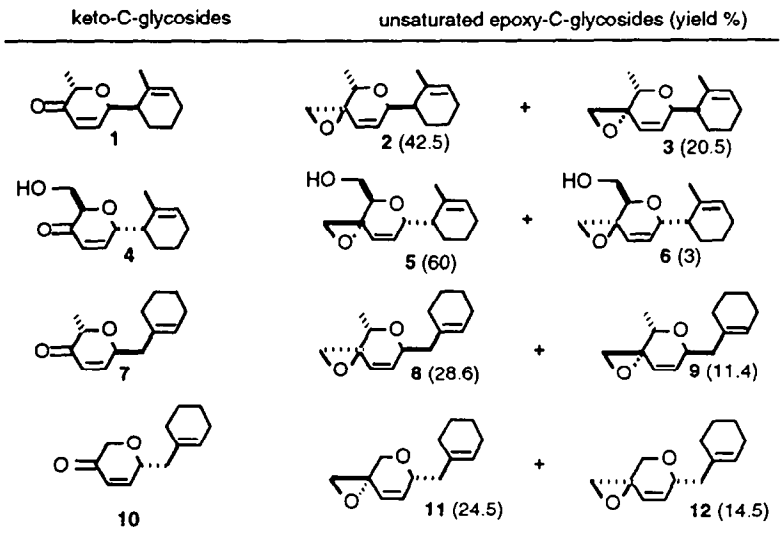
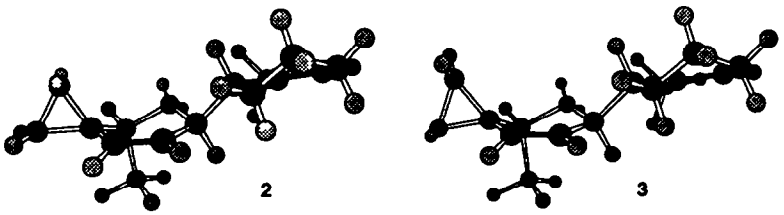


Table I

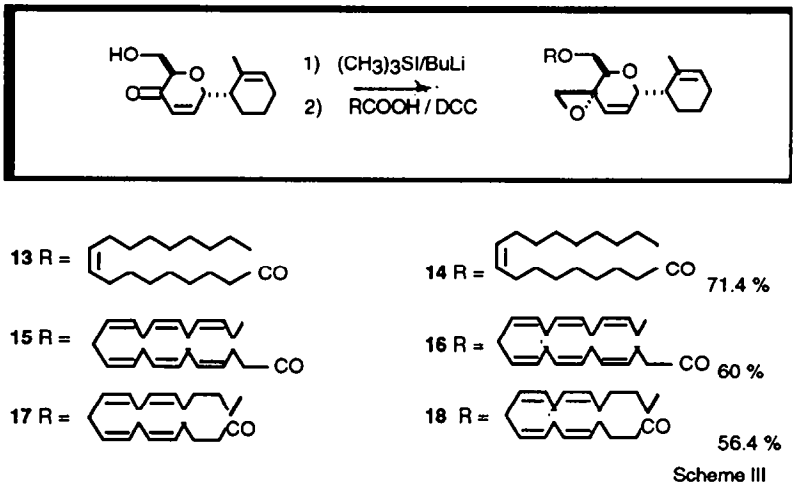
The presence of the epoxy ring was deduced from the ^1H NMR spectra (δ 2.41 and 2.6 ppm). In addition the H-4 signal was observed around δ 5.4 ppm for the major isomers and around δ 6 ppm for the minor isomers. The stereochemistry of the oxirane was established by 1D and 2D NOE experiments combined with molecular modeling. The 2D NOESY spectrum for **2** showed cross peaks between H-6' and H-2 and a correlation between H-6' and the epoxy protons. For the minor isomer **3** H-6' was correlated only to the H-2 signal.

Examination of stereostructures (scheme II) shows that these data are consistent with a $^0H_5$ conformation for the epoxy-unsaturated-C-glycosides and led to the assignation of the C-5 (*S*) configuration for **2** and the C-5 (*R*) configuration for **3**. For the 6-deoxy-L-epoxy-C-glycosides these results and the characteristic chemical shift of the H-4 signal, suggested strongly a C-5 (*S*) configuration for the major isomers (D-epoxy-C-glycosides C-5 (*R*)) and a C-5 (*R*) configuration for the minor isomers (D-epoxy-C-glycosides C-5 (*S*)).



Scheme II Stereostructure of unsaturated epoxide **2** and **3** with use of MM2 and Chem3D

Scheme III summarizes the synthetic approach which was employed to prepare the fatty acid conjugates. First the hydroxy ketone **4** was converted to the unsaturated epoxide then the crude material was treated with oleic, arachidonic or docosahexaenoic acid in the presence of dicyclohexylcarbodiimide[12] to provide the fatty acid ester (yields 56-71 %).



Scheme III

The results obtained for the *in vitro* cytotoxicity experiments[13] against Raji cells, a cultured malignant cell lines derived from a human lymphoma, and LFCL2A cells, a cell line derived from a rat hepatocarcinoma are presented in Table II. Generally, the introduction of the epoxy function at C-5 led to the loss of the cytotoxic activity. However methylene cyclohexene derivatives **8** and **9** exhibited a moderate activity with some differences between the two isomers. The effect of the fatty acid was studied against Raji cells. The oleic acid analogue was inactive while its arachidonic congener was active at the dose of 147.2 μM. Finally the docosahexaenoate exhibited the highest cytotoxicity and was slightly more active than the corresponding keto-C-glycoside **15**. The pattern of structure/activity relationship that emerged from this study demonstrates that the replacement of the carbonyl compound by an epoxide led to the loss of the cytotoxicity. However, when the C-glycosides were bound to the

Table II In vitro cytotoxicity

	Raji		LFCL2A	
Product	IC_{50} (μM)	IC_{50} (μg/ml)	IC_{50} (μM)	IC50 (mg/ml)
1	82.3	17.12	31	6.53
2	NT*		NT*	
3	NT*		NT*	
4	280	62.24		
5	255	60.26		
7			25.2	5.24
8			128	28.3
9			155	34.3
10			26	5.1
11			NT*	
12			NT*	
13	NT*			
14	NT*			
15	62	0.117		
16	58	0.106		
17	66	0.129		
18	147.2	0.283		
19	NT*			
chlorambucil	298			
5-deoxy fluoro uridine	NT*			

* The products were tested until the concentration of 200 μM without exhibiting any cytotoxicity

arachidonic or the docosahexaenoic acid a moderate to high cytotoxicity could be observed especially for docosahexaenoic acid. With respect to the nature of the fatty acid we found that arachidonates were the most active conjugate when the drug was directed toward cellular enzymes whereas docosahexaenoates were more potent when the conjugate was targeted against DNA.

It has been suggested recently, that diverse antitumor drugs may induce a mode of cell death with the characteristics of apoptosis, a natural phenomenon that has been conceptualized as a "programmed" cell death.[14-17] The property of cancer chemotherapeutic agents to induce apoptosis is likely to be due to their capability to artificially stimulate one of the physiological apoptosis pathways. Apoptosis is characterized by cell shrinkage, chromatin condensation and systematic DNA cleavage. The latter is one of the earliest steps and the only known significant biochemical event that occurs during the apoptotic process[18]. In line with this we have determined with a flow cytometric assay[19] the ratio of apoptotic, viable and necrotic cells after treatment of cultures with different drugs.

The results are shown in Figure I and indicate that the percentage of apoptotic cells:

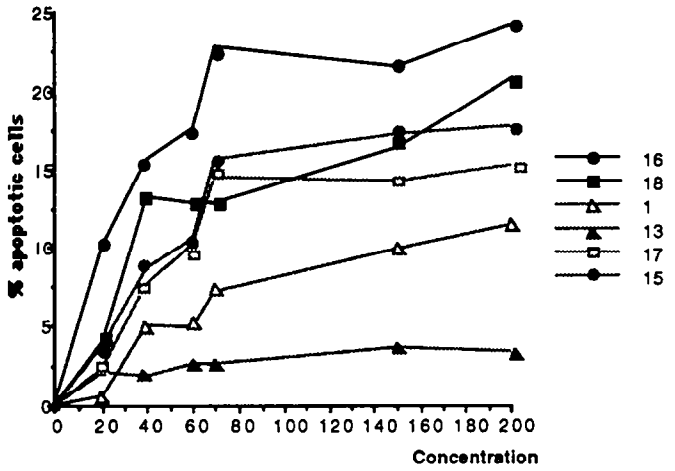


Figure I Percent of apoptotic cell after traitement with C-glycosides

a) is dramatically affected by the coupling with a polyunsaturated fatty acid (compare 1 and 15).

b) is greater with those compounds that possess an epoxide group in place of the keto function (compare 15 and 16).

c) increases with the unsaturation degree of the conjugated fatty acid (see 13 , 15 and 17).

d) shows that there is no relationship between the cytotoxic potential of the drugs and their ability to induce an apoptotic response in the cell (see product **18** Table II and Figure I).

On the other hand the apoptotic effect of epoxides demonstrated here, may result from a direct reaction with DNA, since DNA cleavage is considered characteristic of an apoptotic pathway. This conclusion is consistent with the recent finding that vinyl epoxides have DNA cleavage properties.[9] Finally the results presented here also confirmed that docosahexenoate of DNA alkylating drugs have the highest cytotoxic activity *in vitro*.

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13 LFCL2a and RAJI cells were grown in RPMI supplemented with 10% Fetal Calf Serum (FCS) 1% glutamine and without antibiotics. They were usually seeded at 250.000 cells/ml medium, incubated at 37°C in a humidified atmosphere of 5% in CO_2 and subcultured twice a week. Drugs were kept freeze at -20°C and freshly dissolved in dimethyl sulphoxide (DMSO) at a final concentration of 0.2% in culture medium. Cells, in exponentional growth, were incubated with the drugs over a range of concentrations for 48h at 37°C.

RAJI cells - The cells were then washed twice with PBS and pelleted by centrifugation for 10 min at 120 g. They were resuspended at a density of 10^6 cells/ml in PBS. Propidium Iodide (PI) was added during 5 min. after which the cells were examined by flow cytometry in a Epics Profile Analyzer II (Coulter).

LFCL2A cells - Cell viability was determined by a modified MTT (dimethylthiazolytetrazolium bromide) colorimetric assay. After incubation with the drugs, cells were washed twice and centrifuged for 5 min. at 120 g. They were incubated 3-4 h at 37°C in RPMI supplemented with 2% FCS and 10% MTT. The formazan crystals produced by the cells were solubilized in DMSO and measured spectrophotometrically at 540 nm using a microculture plate reader (titertek Multiskan)

The results were expressed as the drug concentration which inhibited 50% of cell growth. Each experiment was conducted in quadriplicate and the data are the mean of at least three independent experiments.

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