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SYNTHESIS OF A WATER-SOLUBLE ANALOG OF 6-METHYL-3-N-ALKYL CATECHOL LABELED WITH CARBON 13: NMR APPROACH TO THE REACTIVITY OF POISON IVY/OAK SENSITIZERS TOWARD PROTEINS

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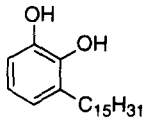
Abstract: A 13-C labeled water soluble derivative of alkylcatechol was synthesized and reacted with human serum albumin in phosphate buffer at pH 7.4 in air to allow a slow oxidation of the catechol into orthoquinone. The formation of several adducts was evidenced by a combination of ^{13}C and ^{1}H-^{13}C correlation NMR. Although some adducts could result from a classical *o*-quinone formation - Michael type addition, our results suggest that a second pathway, involving a direct reaction of a carbon centered radical with proteins could be an important mechanism in the formation of modified proteins.

3-n-Alk(en)ylcatechols, mainly containing 15- and 17-carbon alk(en)yl chains, have been known for many years[1,2] to be the major skin sensitizers in *Rhus radicans* L. (poison ivy), *Rhus diversiloba* Torr. & Gray (poison oak), *Rhus vernix* L. (poison sumac) and *Rhus verniciflua* Stokes (Japanese lacquer tree). These plants, widely spread in North America, Japan, and central and western China, contain an oleoresin, "urushiol", in which alkylcatechols **1** predominate and are responsible for severe allergic contact dermatitis (ACD) reactions; approximately 50-60% of Americans are reported to suffer from ACD due to poison ivy.[3] It has been suggested that these molecules are not real allergens, able to modify skin proteins, but rather pro-haptens[4] that require an oxidation step to be transformed into reactive *o*-quinones **2**, the actual allergen.



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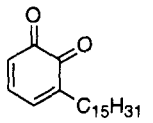

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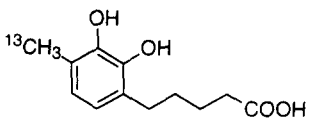

3

In the 80's, the reactivity of such 3-n-alkylcatechols was investigated and it was shown that the addition of amino or thio groups to the derived *o*-quinones was regiospecific,[5] amino groups reacting at the C-5 position and thio groups at the C-6 position of the aromatic ring. Moreover, it was found that the introduction of a methyl group at the C-5 position in order to block the nucleophilic addition of amino groups transforms the allergenic pentadecylcatechol into a skin tolerogen,[6] while the introduction of a methyl group at the C-6 position has little

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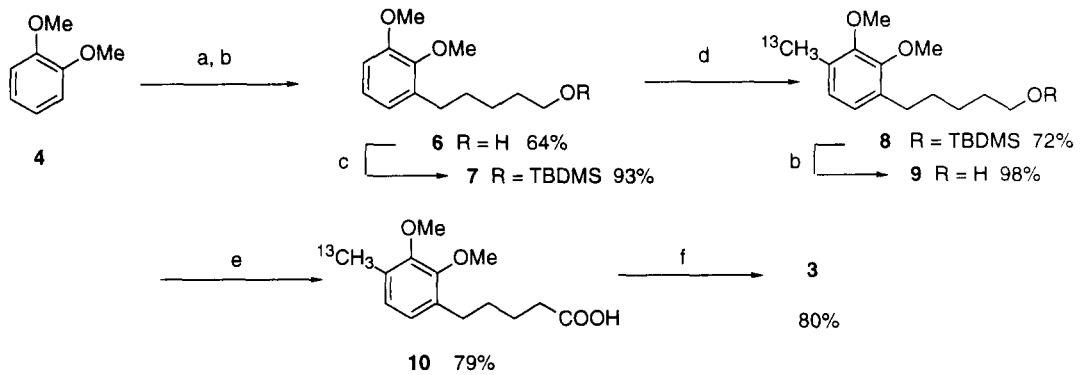
effect on the in vivo reactivity.[7] More recently, orthoquinones have received much interest as key intermediates in the biological activity of estrone derivatives,[8-11] and the behavior of these intermediates has been shown to be complex. In the presence of model nucleophiles in organic solvents, a variety of adducts can be formed, together with self-condensation and reduction products, but very little is known about the reactivity of such quinones toward proteins in aqueous media.

The use of carbon 13-labeled molecules in association with NMR techniques is a powerful tool for the investigation of hapten-protein interactions[12,13] but best results are generally obtained using direct labeling at the reactive site of the molecule. In the case of 3-n-alkylcatechols, such specific labeling at the C-6 and/or C-5 position of the aromatic ring seems to be complex, and we were therefore interested in seeing if a methyl group at the C-6 position could be used as a "reporter" group to investigate the reactivity of such orthoquinones toward proteins in aqueous media. We have previously shown that the use of a DEPT sequence allows the amplification of the CH_3 signals with respect to CH_2 and CH and the suppression of signals due to quaternary carbons, which are abundant in proteins. It is thus possible to directly obtain the signal of the [^{13}C] methyl without having to subtract the residual protein spectrum. Moreover the large range of chemical shifts of methyl groups in both ^{13}C and ^{1}H is the most favorable for a precise identification of substitutions.

In order to facilitate preliminary NMR experiments, we synthesized a water-soluble analog of poison ivy/oak sensitizers **3**, using a shorter alkyl chain and introducing an hydrophilic carboxylic group (Figure 1). We here report the synthesis and preliminary NMR study of this analog with a ^{13}C-labeled methyl group at the C-6 position.

Synthesis: The ^{13}C-labeled catechol derivative was prepared according to the following synthetic pathway[14] (Scheme 1).



Scheme 1: (a) n-BuLi, THF then I-$(CH_2)_5$-OTBDMS; (b) TBAF, THF; (c) TBDMSCl, imidazole, DMF; (d) s-BuLi, TMEDA, Et_2O then [^{13}C]MeI; (e) PDC, DMF; (f) BBr_3, CH_2Cl_2, -78°C.

The intermediate **8** was prepared from veratrol **4** by treatment with n-BuLi in THF to give an ortho-lithiated intermediate[15] that was reacted with 5-iodo-t-butyldimethylsilyloxypentane **5**, prepared in two steps from 5-bromopentanol.[16,17] To facilitate and improve the purification of this intermediate, the silyl protective group was quantitatively removed by treatment with TBAF[18] in THF and alcohol **6** was then reprotected using *tert*-

butyldimethylsilyl chloride (TBDMS-Cl) under standard conditions (DMF, imidazole)[18] to give **7** with a 93% yield.This compound was subjected to a second ortho-lithiation reaction by treatment with s-BuLi and TMEDA[19] in ethyl ether, and the resulting anion trapped using 1 equiv. of [^{13}C] methyl iodide to give intermediate **8** with a 72% yield, together with some starting material. The same reaction carried out in THF, usually used for such o-lithiation reactions, gave poor yields of methylated compounds and most of the starting material was recovered unchanged. The silylated alcohol **8** was classically deprotected by reaction with TBAF in THF and subsequently oxidized with pyridinium dichromate[20] to give the acid **10** with a 79% yield. The methoxy groups were then removed[21] by treatment with 3 equiv. of BBr_3 in CH_2Cl_2 at -78°C to give the catechol **3** with a 80% yield[22] after purification by Sephadex LH-20 gel filtration using degassed solvents. This sensitive catechol was stored at -20°C under an atmosphere of dry argon.

Reaction of the 6-[^{13}C]methylcatechol derivative 3 with HSA: We first determined the chemical shift of the [^{13}C]methyl groups in both the catechol **3** and *o*-quinone **11** forms in a mixture of degassed (to avoid oxidation of the catechol) phosphate buffer/D_2O at pH 7.4.

Compound **3** was dissolved in the solvent mixture and half was oxidized with sodium periodate, then both samples were analyzed by ^{13}C NMR using the same signal amplification sequence DEPT 135. The methyl signal of a trace of acetonitrile was used as the internal reference at 1.32 ppm and the [^{13}C] methyl signal was recorded at 15.5 ppm and 14.7 ppm for the catechol and orthoquinone forms, respectively (Fig 1 A and 1 B). The same operation was carried out in the presence of HSA at the concentrations used for incubation experiments. Although the presence of HSA had very little effect on the chemical shift of the [^{13}C] methyl signal of **3** (Fig 1C, 15.7 ppm), it had a marked effect on that of the *o*-quinone form **11**, with two peaks being seen (Fig 1D), one at 15.7 ppm, resulting from the reduction of the *o*-quinone **11** to the catechol form **3**, and a broad peak at 13.2 ppm, probably due to non-covalent interactions between the *o*-quinone and the protein.

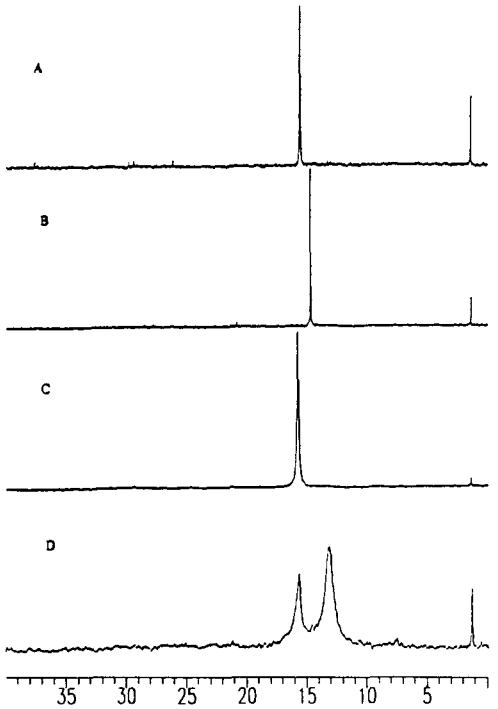


Figure 1: A; ^{13}C NMR of compound **3** in a 1:1 mixture of phosphate buffer/D_2O at pH 7.4. B; ^{13}C NMR of compound **3** oxidized with sodium periodate in a 1:1 mixture of phosphate buffer/D_2O at pH 7.4. C; ^{13}C NMR of compound **3** in a 1:1 mixture of phosphate buffer/D_2O at pH 7.4 in the presence of HSA. D; ^{13}C NMR of compound **3** oxidized with sodium periodate in a 1:1 mixture of phosphate buffer/D_2O at pH 7.4 in the presence of HSA.

Compound **3** (100 molar excess) was then incubated with HSA in phosphate buffer at pH 7.4 in contact with air to allow the slow oxidation of the catechol into its *o*-quinone form and the reaction with nucleophilic residues of the protein. The reaction mixture was then dialyzed and lyophilized, the protein dissolved in a mixture of phosphate buffer/D_2O at pH 7.4 and the ^{13}C{^{1}H} DEPT 135 spectrum recorded (Fig 2). Four sets of signals with very different chemical shifts were detected. The first consisted of two peaks at 9.1 and 9.2 ppm, the second

of one rather broad peak at 12.9 ppm, the third of a large peak at 15.5 ppm, and the fourth of one peak at 23.1 ppm.

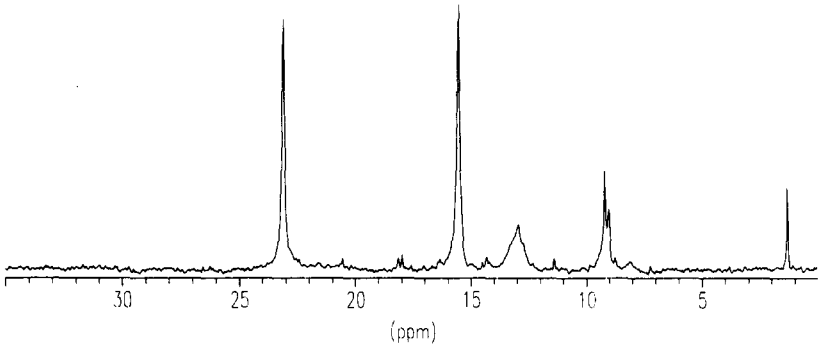


Figure 2: $^{13}C\{^{1}H\}$DEPT 135 spectrum of HSA incubated with compound **3** in air in phosphate buffer.

To obtain more information on both the ^{1}H and ^{13}C chemical shifts, we performed a ^{1}H-^{13}C heteronuclear single quantum correlation (HSQC). This experiment (Fig 3) confirmed the presence of single signals at 9.1/1.27 ppm, 9.2/1.57 ppm (Fig. 3 A) and 12.9/1.7 ppm (Fig. 3 B, broad signal). Moreover, the ^{13}C NMR signals around 15.5 and 23 ppm appeared to correspond to multiple ^{1}H chemical shifts, indicating the presence of several adducts. Thus, the ^{13}C signal at about 15.5 ppm corresponded to 3 adducts (Fig. 3 C) (15.4/2.18 ppm, 15.5/2.30 ppm and 15.5/2.36 ppm), while the single ^{13}C peak at 23.1 ppm corresponded to four adducts (Fig. 3 D) at 23.0/0.48 ppm, 23.1/0.71 ppm, 23.2/0.86 and 22.9/0.34 ppm.

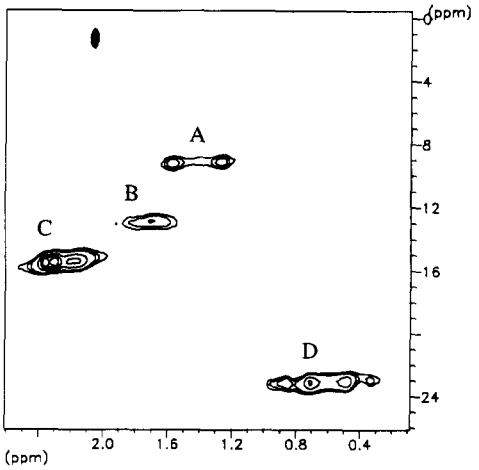
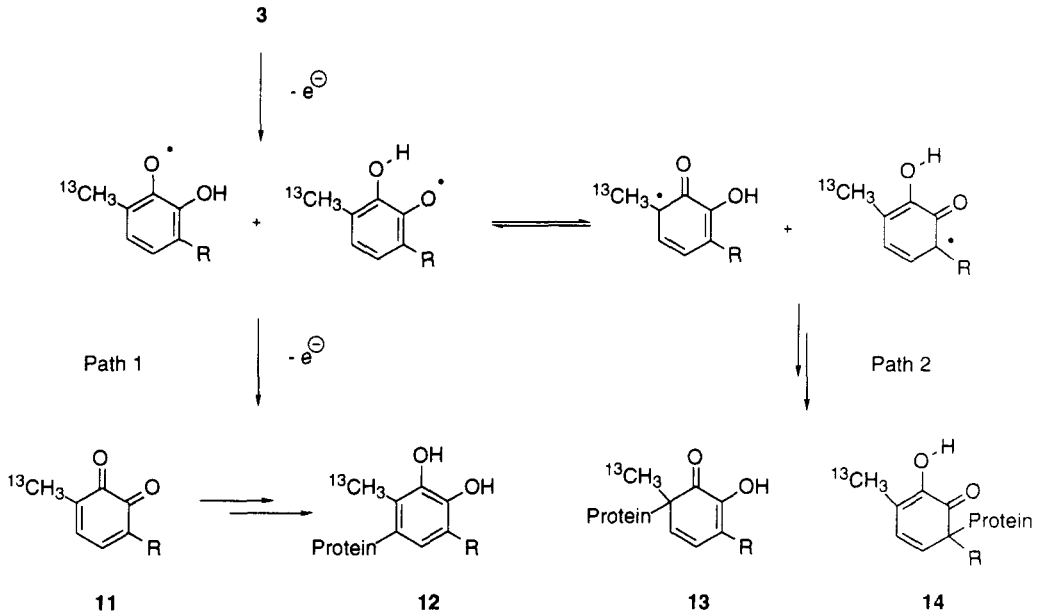


Figure 3: HSQC of HSA incubated with compound **3** in air at pH 7.4 in phosphate buffer.

Reactivity of orthoquinone derivatives: As pointed out in previously published studies, the chemistry of *o*-quinone derivatives is complex.[5-11] Based on the additivity principle, it is possible to calculate the theoretical ^{13}C chemical shift of the labeled methyl group at position 6 of the aromatic ring. These values only take into account intrinsic electronic parameters and do not integrate potential shifts induced by the environment of the protein. On the basis of these calculations, it appeared that the introduction of any heteroatom (N, O, S etc) at the C-5 position of the aromatic ring led to a decrease in chemical shift of about 5 ppm, while the introduction of any heteroatom at the C-4 position as well as the formation of a Schiff base at the C-1 or C-2 position led to no significant modification in chemical shift of the methyl group. Therefore the peaks seen at 9.1 and 9.2 ppm (Fig. 3 A) could arise from a classical Michael type addition of amino groups on the intermediate *o*-quinone **11** (Scheme 2, path 1) at the C-5 position, as previously reported[5] while the broad signal at 12.9 ppm (Fig. 3 B) could be due to a residual *o*-quinone sign.

The signals at about 23 ppm (Fig. 3 D) are much interesting, as such an increase in chemical shift (+ 8 ppm) is very difficult to explain on the basis of classical addition models; it could arise either from a large change in

electron density on the aromatic ring or from the location of methyl groups in a deshielding zone of the protein. However, the deshielding explanation should apply to both ^{13}C and ^{1}H, but the corresponding ^{1}H chemical shifts, with values of 0.34, 0.48, 0.71 and 0.86 ppm, respectively, do not support this hypothesis. Moreover, it would be surprising to observe 4 different adducts in a similar deshielding zone. On the other hand, the electronic explanation is not compatible with classical 1,2 or 1,4 addition of any nucleophilic amino acid on the intermediate *o*-quinone **11**, since all proposed structures would lead to an equal or decreased ^{13}C chemical shift for the methyl groups.



Scheme 2: Possible reaction pathways of **3** with HSA in phosphate buffer at pH 7.4 in the presence of oxygen

The only possibility that would lead to a marked increase in the ^{13}C chemical shift is the formation of a carbon-heteroatom bond or preferably a carbon-carbon bond at position 6, which implies a break in ring aromaticity, to give adducts **13**. This unusual hypothesis is strongly supported by the observed methyl group ^{1}H chemical shifts (0.4 to 0.8 ppm), which are more compatible with an alkyl rather than an aromatic structure. It would be surprising if such adducts at position 6 were formed by a classical Michael type addition, even though this is theoretically possible, as this position is very much hindered and in competition with more accessible reactive sites. We therefore suggest that such adducts could be formed via a radical mechanism (Scheme 2, path 2), following the first step of electron transfer in the oxydation of catechols into *o*-quinones. Reactions on ortho positions are then favored by the presence of alkyl groups which stabilize the carbon centered radicals. Such a mechanism could also occur at position 3 of the catechol and would give adducts **14** with chemical shifts compatible with peaks observed at about 15.5 ppm (Fig. 3 C). Nevertheless, the modification being far from the ^{13}C probe, this assignment is not conclusive and could also correspond to a mixture of the starting catechol **3** and hydration or adduct compounds at position 4. The formation of a Schiff base at the C-1 or C-2 position, which could lead to similar chemical shifts for the methyl group, was ruled out by the reduction[22] of the modified HSA

with 100 equiv. of $NaBH_4$ in borate buffer at pH 9.3. No modification could then be observed in chemical shifts of methyl signals.

Conclusion. Reaction of catechol **3** with a model protein in water at pH 7.4 and in the presence of oxygen led to the formation of several adducts as demonstrated by a combination of ^{13}C and ^{1}H-^{13}C correlation NMR. Although some adducts could result from a classical *o*-quinone formation - Michael type addition, usually considered to explain the sensitizing potential of poison ivy/oak catechol, our results suggest that a second pathway, involving a direct reaction of a carbon centered radical with proteins and occuring after the first electron abstraction of the oxydation process, could be an important mechanism in the formation of modified proteins. This approach provides new insights into both the nature of the modified amino acids and thus the generation of T-cell epitopes, and the modification position on the aromatic ring.

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14. All compounds were fully characterized and gave satisfactory microanalysis.
    Compound **3**: white solid; mp = 115-116°C. ^{1}H NMR (200 MHz, acetone d6) δ 1.59-1.70 (m, 4H, CH_2), 2.17 (d, 3H, J_{C-H} = 126.4 Hz, Me), 2.31 (m, 2H, CH_2-COOH), 2.60 (m, 2H, CH_2-Ar), 2.89 (bs, 2H, OH), 6.53-6.59 (m, 2H, H_4 + H_5). ^{13}C NMR (50 MHz, acetone d6) δ 16.0, 21.6, 25.5, 30.3, 34.1, 121.3, 121.4, 122.0 (d, J_{C-C} = 44 Hz), 127.7, 143.9 (2C), 174.8. IR ($CHCl_3$) ν 3604-3529-3368-3238 (OH), 1707 (C=O). Anal. Calcd for $C_{11}{}^{13}CH_{16}O_4$: C, 64.43; H, 7.16. Found: C, 64.73; H, 7.09.
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