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Dithranol reaction with nitroxide radicals in DMSO, a HPLC study

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Dithranol (1,8-dihydroxy-9-anthrone), an efficient drug for the topical treatment of psoriasis undergoes a complex chemical transformation after topical application. An absorption phase HPLC method has been developed and validated to follow the appearance of its oxidative products in a DMSO solution. In DMSO solution dithranol, chrysazin, and biantrone were monitored simultaneously by HPLC during autooxidation process, as well as in the presence of nitroxide radicals which increase the reaction rate. The kinetics of the very early stage of dithranol transformation is presented for the first time and discussed. Two unknown dithranol-derived intermediates were found and partially characterised.

1. Introduction

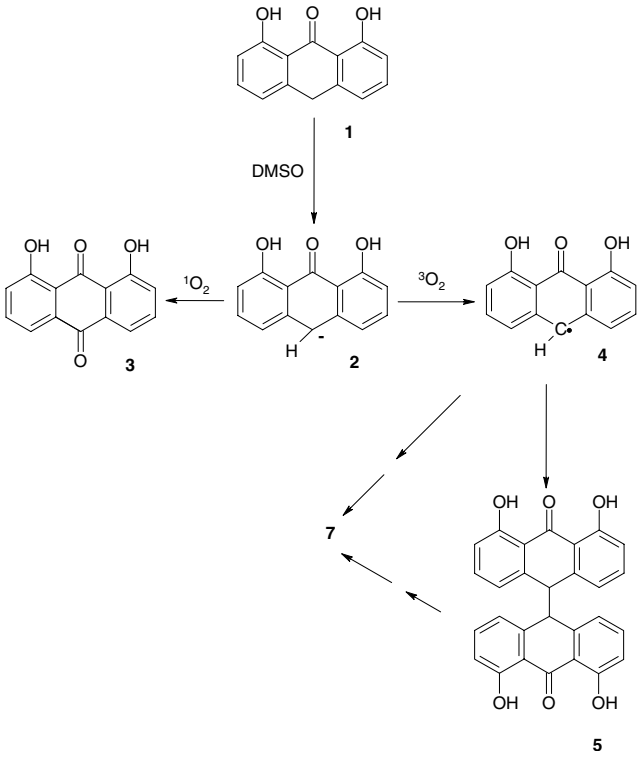
The prevalence of psoriasis in developed countries is approximately 2%. Due to its resistance to medication and its long term of duration, it seriously affects the quality of life [1]. Among antipsoriatic drugs, dithranol (1,8-dihydroxy-9-anthrone, anthraline, **1**) has been used successfully for decades, but its mechanism of action has not yet been defined [2]. The main reasons are the facts that the pathogenesis is unexplained and that the disease is known only in humans. Authors in most recent publications agree that the formation of a 10-dithranyl radical (1,8-dihydroxy-9-anthron-10-yl, **4**) should be important for the biological effects, but it is still not clear whether **4** itself or the subsequently formed secondary radicals are the active species [3–7].

It is known that **1** (Scheme 1) in DMSO or acetone solution in the presence of air slowly reacts with oxygen (autooxidation) via its anion (**2**) to a complex cross-linked dark polymer known as dithranol brown material (**7**) which still contains radicals (Scheme 2). A similar transformation also occurs after topical application of ointment containing **1** as a drug. In this case, the dark paramagnetic material is covalently bound to the skin [3]. All these radicals have been established [5–7].

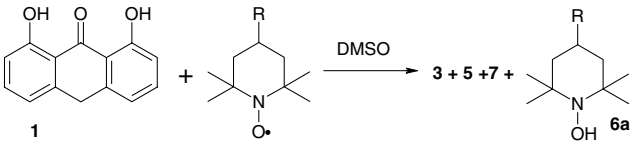
In a model system (water, or organic solvents like xylene, acetone, pyridine, DMSO) radical **4** can be observed, identified and trapped [7–9]. UV irradiation, alkaline conditions, oxidants, and heating above 100 °C in inert solvent without oxygen also leads to formation of **4**, which has been detected and characterised directly by EPR spectroscopy [6, 10]. This radical should be a reactive intermediate in further complex oxidation reactions where superoxide and hydroxyl radicals are observed. It is now accepted that the formation of **2** is the first step in the transformation and that the presence of oxygen is crucial for formation of **4** and for continuation of the reactions, while traces of metal ions accelerate this transformation.

In DMSO solution under aerobic conditions **1** is slowly converted into stable chrysazin (**3**, a minor product), biantrone (**5**, an unstable intermediate), and into a dark brown paramagnetic material which is a final product of the radical oxidation path [11]. DMSO as a weak base helps to form **2** which reacts further with oxygen dissolved in the DMSO [12]. It has been reported that the nitroxide radical (TBz, 4-benzoyloxy-2,2,6,6-tetramethyl-1-oxylpiperidine, **6**) is reduced by **1** in DMSO solution (Scheme 2) into the hydroxylamine **6a** and that **5** is trans-

Scheme 1



Scheme 2



R = -$OCOC_6H_5$ 6
R = -$NHCOC_6H_5$ 6b

formed in the presence of **6** after a short induction period with a rate similar to that for **1** itself. In this EPR study **6** was recognised as oxygen-mimetic compound suitable for studying the dithranol oxidative transformation [12].

Several analytical methods for determining dithranol in different pharmaceutical forms have been described. Due to its ability to be contaminated by its autooxidative products the separation has to be an important element of the analytical method. In addition, the presence of a large quantity of lipophilic ingredients in pharmaceutical formulations also requires the combination of separation and analytical techniques [13–15].

The most recent Pharmacopoeia monographs introduced chromatographic methods (active pharmaceutical ingredient) [16–18]. TLC methods were described, but they were not able to demonstrate the presence of radicals [19]. Fluorescence spectroscopy was also used as a very sensitive method which due to its specificity, does not need so many extraction and separation steps [15].

There are several HPLC methods for the determination of **1** as an active ingredient but most authors state poor resolution between **1** and **3**, and the prolonged retention time of **5** affects the peak shape [13–15].

In this paper we present the results of an HPLC investigation of dithranol autooxidation and the influence of nitroxide radical on this reaction.

2. Investigations, results and discussion

To get a deeper and quantitative insight into the above reactions we have developed and evaluated a HPLC method which separates dithranol effectively from the products of oxidation. The addition of glacial acetic acid to the mobile phase stops the first reaction (Scheme 1) and this enables us to trace the changes of concentration of dithranol (**1**), chrysazin (**3**) and bianthrone (**5**) simultaneously with time. Using this approach we evaluate the kinetics of autooxidation of dithranol in DMSO solution and compare this reaction with the more rapid reaction in the presence of nitroxide radical which replaces oxygen in accepting one electron from dithranol anion. Higher concentrations of nitroxide accelerate the transformation (oxidation) of dithranol. The appearance of products was followed by HPLC. The comparison of the two reactions is discussed, in order to gain a broader insight into the interrelation between the source substances, intermediates, and final products. The observed short lived intermediates were partially characterised.

2.1. HPLC method

The basic adsorption phase of the HPLC method for monitoring compounds **1**, **3** and **5** was described by Sceah et al. for determining of dithranol in pharmaceutical formulations [15]. To study dithranol oxidation we modified both the mobile and stationary phases. Four different stationary phases, i.e.: Porasil (Waters), Silica (Spheri-5), Spherisorb Silica (Phase Sep), and Nucleosil Si 50-5 (Macherey Nagel) have been tested. Nucleosil Si 50-5 was found to be the most efficient under the experimental conditions used.

Standard and sample preparation procedures were developed to overcome the low solubility of DMSO in the mobile phase. To transfer the DMSO reaction mixture into the mobile phase we first added dichloromethane and then the mobile phase. The presence of acetic acid in the mobile phase stopped the reaction between dithranol and any basic species in the reaction mixture. The absence of anion **2** stopped the generation of radical **4** and we assume that most of the other changes in the mixture, including the oxidation by oxygen, were stopped by the addition of acetic acid.

The HPLC method was validated according to USP requirements [17]. Validation was applied as a statistical tool to assure the reliability of the results from the real system. The analytical matrix was spiked with standards of all known compounds to get as close as possible to the real system.

Typical analytical parameters used in HPLC validation were:

Range – the method used covers the range between 0.2 and 40.0 μg/ml.

Linearity – regression lines are strictly linear within the defined range.

Precision – the relative standard deviation (coefficient of variation) is less than 2% with 5 repetitions of a homogenous sample.

Accuracy – recovery of known, added amounts of analyte is greater than 95% (for 1, 4, 10 μg/ml quality control samples).

Limit of quantitation/detection – the limit of quantitation is set at the lowest point of the standard curve, but several times lower concentrations can be detected.

Selectivity – all three analytes are well resolved, as well as two major unknown intermediates. Under the conditions used (354 nm) neither TBz, TABz nor their reduced derivatives were observed in the chromatograms.

The mixture of standards: dithranol (Rt = 4.4 min), chrysazin (Rt = 5.2 min) and bianthrone (Rt = 10.6 min) is well resolved (Fig. 1) under the conditions described.

The retention times for known compounds were acceptable, but due to an unknown, late eluted intermediate, each run took 30 min. In one run we could determine all known compounds and observe the appearance of unknown intermediates as a function of time. Using the calibration curves the amounts of **1**, **3**, and **5** were calculated. The amount of unknown compounds was simply calculated by subtracting **1**, **3**, and **5** from the starting amount of dithranol.

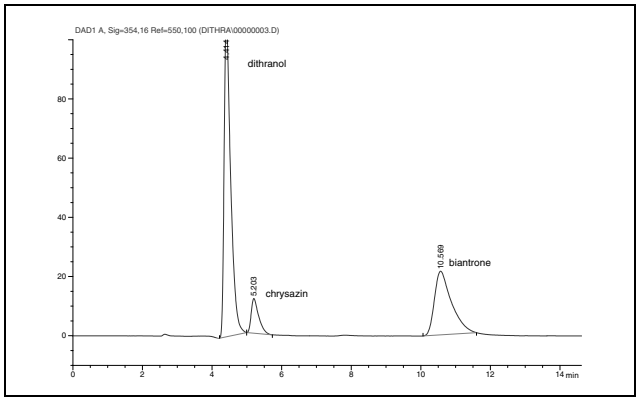


Fig. 1: Chromatogram of mixed standard solution

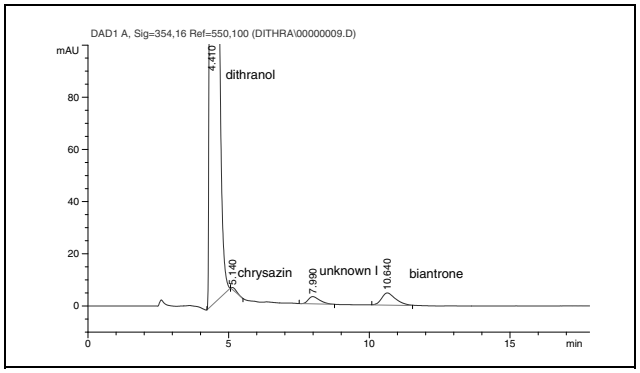


Fig. 2: Chromatogram of the sample – Autooxidation in DMSO after 12 h

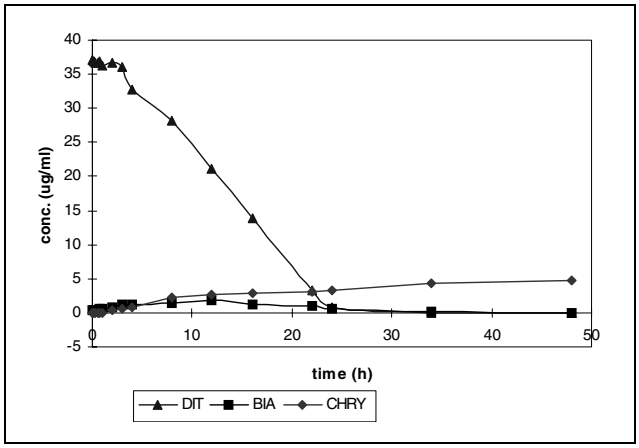


Fig. 3: Autooxidation of dithranol

2.2. Autooxidation of dithranol in DMSO

Dithranol is transformed in DMSO under aerobic conditions by different reactions into stable chrysazin, a paramagnetic end product **7**, which is not involved in further transformation, while **5** is still a reactive intermediate. In the autooxidation reaction in addition to **1**, **3**, and **5**, after 12 h we detected the first unknown intermediate (Rf = 8.1 min) and after 18 h (not shown) the second one (Rf = 23.9 min) (Fig. 2).
The time dependent changes in concentrations of **1**, **3**, and **5** are presented in Fig. 3. Dithranol concentration stayed at the initial level for the first 3 to 4 h, after which its concentration began to decrease. It dropped to one half after about 14 h and almost to zero after 34 h. After the "induction period" the decrease of 1 proceeded with a constant rate 1.75 μg/ml h (7.7 nmol/ml h).

After 2 h the presence of **3** was detected and its concentration continued to increased gradually all the time from 0.3 μg/ml to 4.7 μg/ml at 48 h (from 1.25 μmol/l to 19.58 μmol/l). According to published data the appearance of **3** is a consequence of dithranol oxidation by singlet oxygen [11]. The increasing concentration of **3** could be the result of action of singlet oxygen but other reactions are not excluded.
Bianthrone appeared at the very beginning of the reaction and its maximal concentration 1.9 μg/ml (42 μmol/l) was reached after 12 h. It then slowly dropped to zero (48 h). The kinetics of bianthrone is typical for an intermediate.

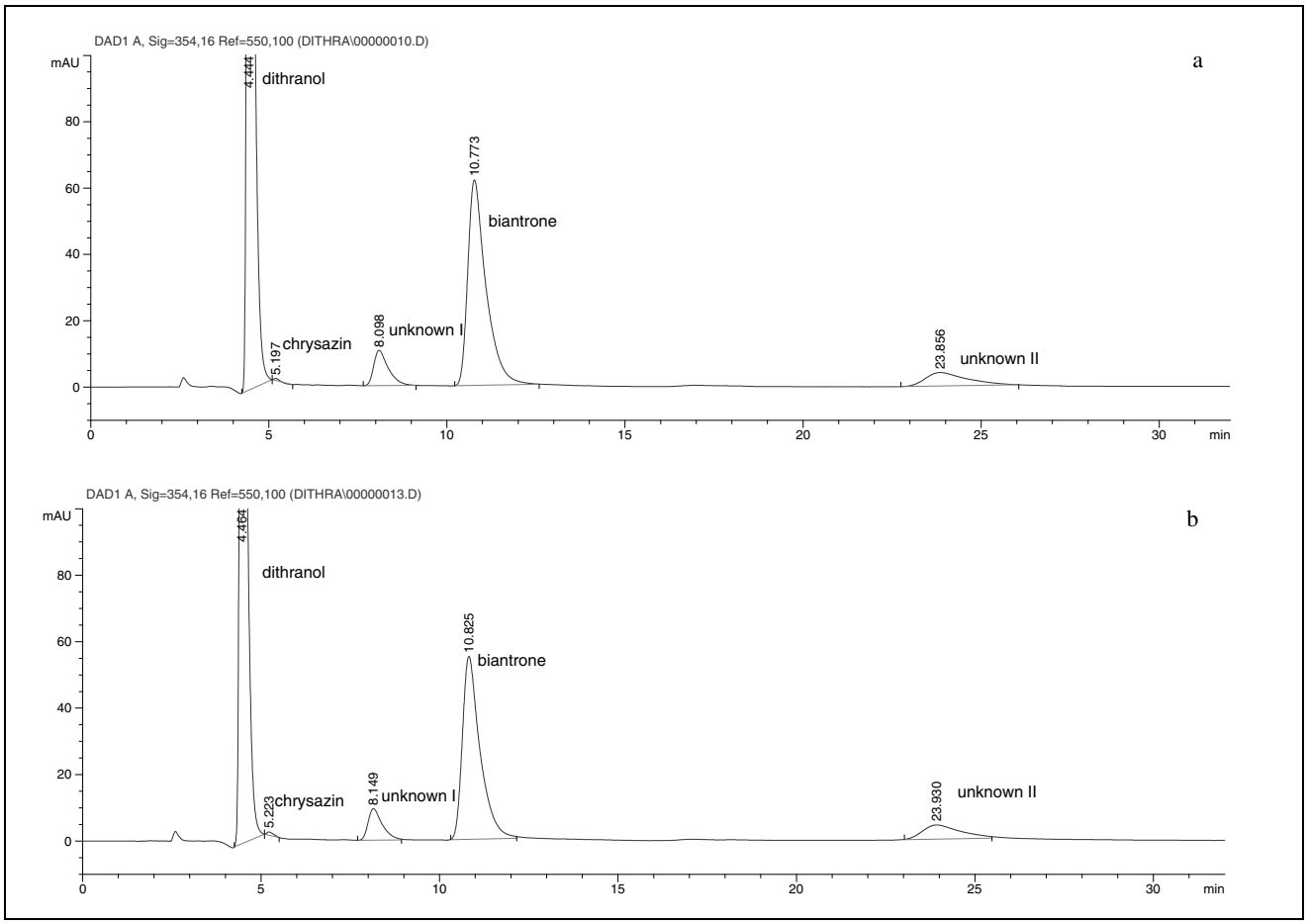


Fig. 4: Chromatogram of the sample – reaction with nitroxide in 1 : 1 molar ratio after 1 h a) TBz b) TABz

2.3. Reaction of dithranol with nitroxides

We found that the addition of nitroxide into the reaction mixture increases the rate of dithranol oxidation to **5** and **7** (Fig. 4a, 4b). Two nitroxides with different lipophilicity were probed: TBz (R=OCOPh) and TABz (R=NHCOPh). The more hydrophilic amide nitroxide (**6b**, TABz) was eluted (free radical as well as reduced hydroxylamine form) after all the compounds of interest. However, the less hydrophilic TBz (ester) still had good selectivity. The reaction rate in both cases was practically the same. Therefore in further experiments we used only TBz.

In the presence of one molar equivalent of **6** the concentration of **1** was reduced to 50% in 15 min (Fig. 5). At this point the reaction slowed down. The slope of the concentration curve of **1** after 15 min was similar to that of the autooxidation curve slope after the "induction period" (3–4 h). This shows that all the TBz was used in the reaction with dithranol, whose further transformation become again oxygen dependent. However the molar ratio of the reaction between dithranol and nitroxide appears to be complicated and it is greater than 1 : 1.

The appearance of 3 was delayed for 2 h, as in the autooxidation reaction. After 48 h its concentration was 2.3 μg/ml (9.58 μmol/l), which was approximately 50% of the amount observed in the autooxidation reaction. In the presence of nitroxide the formation of **3** was less pronounced.

Bianthrone appeared at the beginning of the reaction in much higher concentration. The maximal level 9 μg/ml (20.0 μmol/l) was reached after 15 min and then decreased to zero 22 h. The highest concentration of **5** was approximately five times larger than in the case of autooxidation. The presence of nitroxide in the reaction mixture led to the generation of significantly larger amounts of **5** in a shorter time, as in the case of autooxidation. Nitroxide probably acts as an electron acceptor (like oxygen but more potent) which quickly shifts the anion **2** into the radical form **4** by one electron transfer. Higher concentrations of **4** led to higher concentrations of **5** which transformed further, probably in a different manner.

The profile of the reaction in the presence of nitroxide in the period between 15 min and 1 h was comparable to the profile of the autooxidation reaction in the period between 12 and 24 h. A difference was observed in the profile of degradation products and intermediates. In autooxidation there were lower concentrations of **5** and of both unknown intermediates, but **3** was found in higher concentration.

The same unknown intermediates were also seen in the case of the reaction of dithranol with TBz (Fig. 4a) or TABz (Fig. 4b), however, no significant differences were observed using a different nitroxide. Their concentrations were than under the autooxidation conditions.

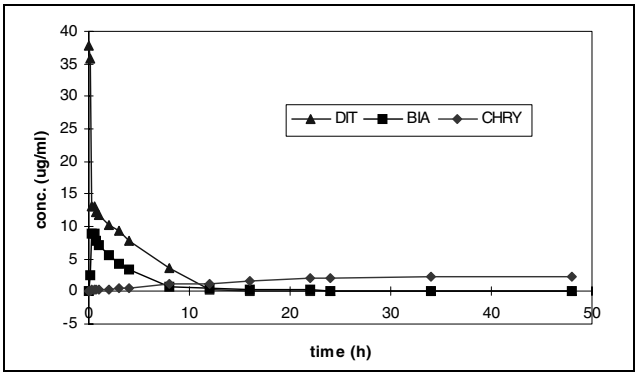


Fig. 5: Reaction of dithranol with nitroxides in 1 : 1 molar ratio

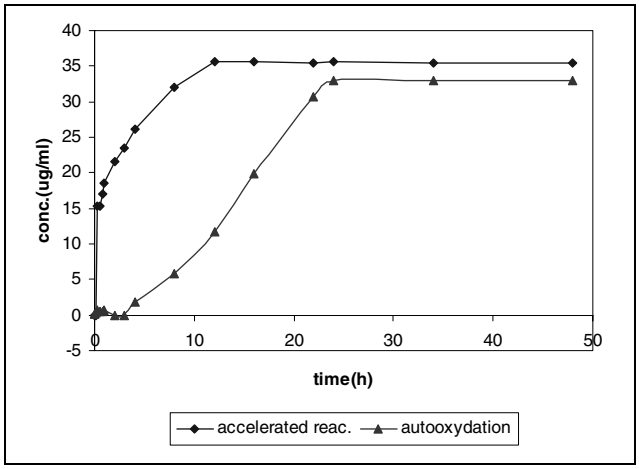


Fig. 6: Appearance of dithranol brown and unknown products (calculated value)

2.4. Partial characterisation of unknown intermediates

In both cases (autooxidation and faster reaction) we observed two unknown intermediates. For on-line monitoring we introduced a rapid scanning and diode array option to scan UV spectra (Figs. 7–9). Given the same Rt values and identical UV spectra of intermediates observed in autooxidation and in more rapid reactions the intermediate structure should be very similar in both cases. The UV spectrum of the first unknown intermediate was similar to that of **1**, while the spectrum of the second unknown intermediate was similar to **5**. Spectral peaks were shifted several nm, which could mean that these intermediates were a kind of adduct of dithranol and bianthrone radical with one of the solvent molecules from the reaction mixture. We have already attempted to characterise the unknown intermediates using different analytical approaches but the UV spectra alloved the only succesful characterisation so far due to their very short period of life and very low concentrations. The UV data indicate that for the first unknown intermediate results from the reaction of the dithranol radical **4** with one of the solvent components. This adduct is probably further transformed into an unstable species which can be detected with HPLC, but is not sufficiently stable to be completely characterised. We propose that similar reactions take place with the bianthrone radical, based on the similar UV spectra of the second unknown intermediates which resemble **5**. In both cases, howevers, the oxidative dithranol transformation gives re-

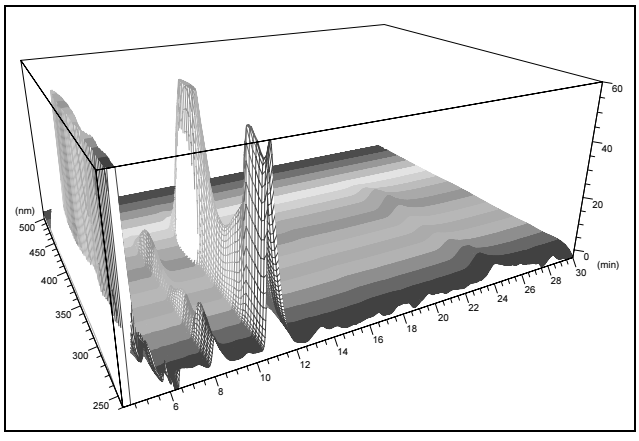


Fig. 7: Chromatogram/spectra of the reaction of dithranol with nitroxides obtained with photodiode array detection

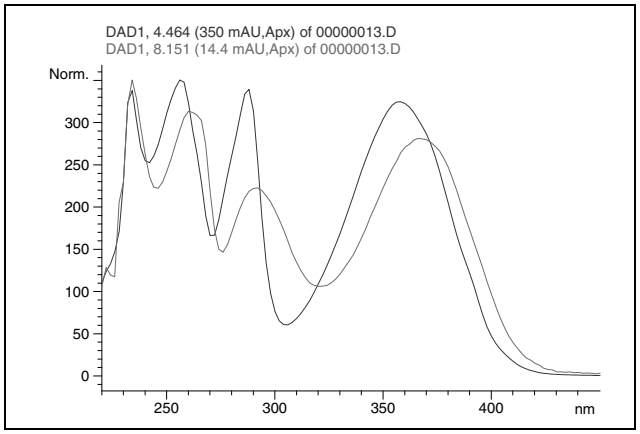


Fig. 8: Spectral comparison of dithranol and the first unknown intermediate (Rt = 8.1 min)

active species which are able to react with surrounding molecules. This mechanism supports the reported irreversible binding of dithranol on isolated animal skin 5.

2.5. Conclusions

The behavior of dithranol in DMSO solution is a useful system for simulating the oxidative transformation of dithranol after topical application. This model system was explored by an HPLC analytical approach which enabled simultaneous, quantitative, time dependent monitoring of dithranol and its major oxidative products and intermediates. The rate of oxidation was drastically increased in the presence of nitroxide with respect to the rates observed in autooxidation. We observed little change in the profile of the related compounds but the overall reaction rate increased several fold. This approach was found suitable for sampling and was less time consuming. The appearance of unknown intermediates and their partial characterisation by UV spectroscopy confirmed the reaction of the dithranol radical with surrounding molecule.

3. Experimental

3.1. Instrumentation and equipment

The modular HPLC system (Thermo Separation Products) consisted of Degasser SCM 1000, Pump P 1000, Autosampler AS 1000, Detector UV 2000, Integrator Chrom Jet 4400 and Hewlett Packard (Agilent) modular HPLC system HP 1100 with photodiode array detector and Chemstation rev. A.07.01.(682).

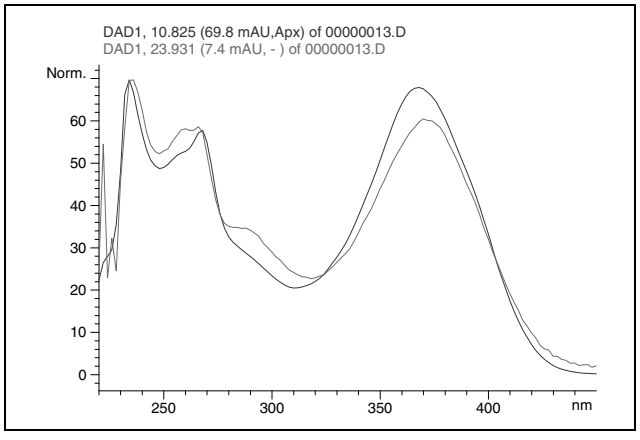


Fig. 9: Spectra comparison of biantrone and the second unknown intermediate (Rt = 23.9 min)

3.2. Chemicals and solvents

Dithranol (**1**) was obtained from Aldrich and purified on a silica gel column (Kieselgel 60, 70–230 mesh, Merck) using dichloromethane as eluent.
Bianthrone (**5**) and chrysazin (**3**) (HPLC standard) were gifts of Prof. Dr. Wolfgang Wiegrebe, Faculty of Pharmacy, Regensburg, Germany
4-Benzoyloxy-2,2,6,6-tetramethyl-1-oxylpiperidine (TBz, 6) and 4-benzoylamino-2,2,6,6-tetramethyl-1-oxylpiperidine (TABz 6b) were prepared at the Faculty of Pharmacy, Ljubljana [20].
DMSO, p.a. grade, isooctane, HPLC grade, dichloromethane, HPLC grade and glacial acetic acid HPLC grade were obtained from Merck.

3.3. Chromatographic conditions

HPLC separation was performed on a 250 × 4.6 mm i.d. stainless steel column, packed with adsorption phase Nucleosil Si 50–5, 5 μm particle size (Macherey Nagel, Duren, Germany). The column was thermostatted at 40 °C during all experiments. The mobile phase was: isooctane (94%), dichloromethane (3%), and glacial acetic acid (3%). Sample injections (20 μl and 100 μl for unknown intermediates) were made using a loop injector and autosampler. Compounds were detected at 354 nm. For partial characterisation of unknown intermediates the rapid scanning option and photodiode array detection (all spectra) with Chemstation software were used.

3.4. Preparation of standards and calibration curves

Stock solutions of **1**, **3** and **5** containing 1 mg/ml were made separately in dichloromethane. The mixed standards were prepared in the mobile phase with the addition of dichloromethane, to 12.7% in the final solutions. Sets of mixed standards were prepared to cover the range from 0.2 to 40 μg/ml and the calibration curves were recorded. Three mixed QCS (quality control samples) were prepared to cover low, medium and high concentration ranges.

3.5. Sample preparation

Ten ml of 33.3 mmol/l solutions of dithranol in DMSO were mixed with 10 ml of DMSO (autooxidation) or 10 ml of 33.3 mmol/l TBz (or TABz) solution in DMSO.
According to the time schedule 1 ml of reaction mixture was put into a 100 ml volumetric flask containing 10 ml of dichloromethane, mixed well and mobile phase added to volume. Solutions were filtered through 0.45 μm filters and 20 μl of filtrate were injected in the stable HPLC system. All reactions and operations were performed under aerobic conditions at room temperature.

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