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Isolation, Chemistry, and Biochemistry of Ptaquiloside, a Bracken Carcinogen

Kiyoyuki Yamada,* Makoto Ojika, and Hideo Kigoshi

Bracken (*Pteridium aquilinum*) is a toxic and yet edible plant which is distributed in many parts of the world. The carcinogenicity of bracken has been a long-standing problem. This review provides an account of the extensive studies on a bracken carcinogen. Development of a new and mild extraction method coupled with a carcinogenicity test for rats as a bioassay led to the discovery and isolation of ptaquiloside; the biological activities of this long-sought carcinogen are described here. The ultimate carcinogen derived from ptaquiloside is so reactive that it not only efficiently alkylates sulfur-containing amino acids and DNA, but also cleaves DNA. The molecular mechanism of this DNA cleavage was determined. Furthermore, both enantiomers as well as artificial analogues of the ultimate carcinogen were synthesized, and their DNA-cleaving activities were evaluated. Related studies on the bracken carcinogen are also briefly described.

Keywords: bioorganic chemistry · DNA cleavage · glycosides · structure elucidation · terpenoids

1. Introduction

The bracken fern, *Pteridium aquilinum* (L. Kuhn), is widely distributed throughout the world and is consumed as a human food in Japan and some other countries. Its toxic effects on livestock have been known since the end of the 19th century; cattle that consumed bracken exhibited cattle bracken poisoning.[1–3] The major feature of this syndrome is the depression of bone-marrow activity, resulting in severe leucopenia, thrombocytopenia, and the hemorrhagic syndrome. The earliest evidence for the carcinogenicity of the bracken fern was reported by Rosenberger and Heeschen, who described the changes in the nature of polypus tumors in the urinary bladder mucosa accompanied by haematuria in cattle that ingested the bracken fern.[4] The carcinogenicity was unambiguously demonstrated by Evans, who reported that rats fed diets containing the bracken fern developed multiple ileal adenocarcinomas;[5] this result was confirmed by many research groups. Furthermore, it was shown that the carcinogenicity of young bracken (Figure 1) was greater than that of mature bracken (Figure 2) and that the carcinogenicity of bracken treated with hot water containing sodium bicarbonate or wood ash remarkably decreased.[2] In spite of the enormous efforts made by many groups in the search for a bracken carcinogen, its isolation was unsuccessful. This was made difficult by two major factors: a) the instability of the

Figure 1. Young bracken used as human food.

Figure 2. Mature bracken.

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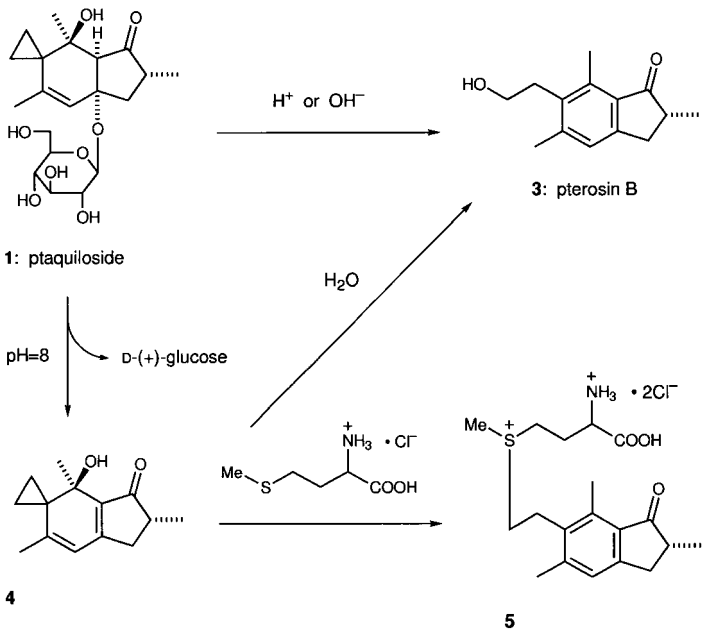
carcinogen and b) the nature of the assay system; various short-term tests for the bioassay were applied without success.[2, 6]

2. Isolation and Biological Activities of Ptaquiloside

In 1978 Hirono reported that an aqueous extract obtained by treatment of bracken with boiling water for three minutes was carcinogenic to rats.[7] We were interested in this result, which clearly showed that the bracken carcinogen could be extracted in boiling water. Therefore, we started to fractionate the aqueous extract of bracken on the basis of the carcinogenicity test for rats, although the test required large amounts of samples and took a long time. After five separation steps employing solvent partitions (water/butanol) and neutral resins (Amberlite XAD-2 and Toyopearl HW-40), the strongly carcinogenic fraction was obtained. This fraction was further separated by repeating normal and reverse-phase chromatography three times to result in the isolation of an unstable new compound termed ptaquiloside (**1**, Scheme 1), which later proved to be the long-sought carcinogen of bracken.[8, 9]

Ptaquiloside was obtained in low yield (0.2 g from 1 kg of bracken powder) by the bioassay-guided fractionation procedure, as most of the product decomposed, while it was isolated in much better yield (ca. 1 g from 1 kg of bracken powder) by the improved extraction method.[10] The development of a new and mild extraction method in conjunction with the use



Scheme 1. Reactions of ptaquiloside (**1**) and the ultimate carcinogen **4**.

of the carcinogenicity test for rats as a bioassay was the key to the successful isolation of **1**. In the same year (1983) that we isolated **1**, van der Hoeven et al. reported the isolation and gross structure of a mutagenic compound named aquilide A from bracken which seemed to be identical to ptaquiloside.[11]

The biological activities of ptaquiloside were evaluated in detail. Thus, **1** was unambiguously shown to be a carcinogen;

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tumors developed in both the ileum and the urinary bladder, target organs of bracken carcinogenicity, of all rats given diets containing **1**.[12, 13] It was also demonstrated that **1** causes cattle bracken poisoning,[14] and it proved to be responsible for bright blindness, progressive retinal degeneration (PRD), in sheep.[15]

3. Structure and Reactivity of Ptaquiloside

3.1. Structure

Ptaquiloside (**1**) is a colorless amorphous compound with the molecular formula $C_{20}H_{30}O_8$.[8, 16] In aqueous solution **1** gradually converts into pterosin B (**3**)[17] and D-glucose. The presence of the partial structures A – G (Figure 3), which

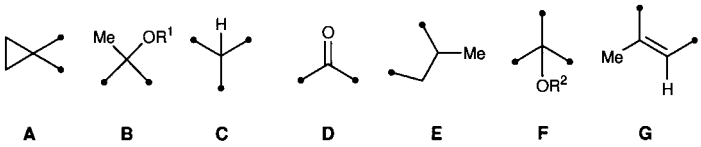
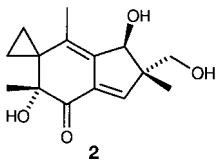


Figure 3. Partial structures of ptaquiloside (**1**).

contain all the carbon atoms of the aglycon part of **1**, was revealed by ^1H and ^{13}C NMR spectroscopy. However, the correlation of these partial structures was difficult with the NMR spectroscopic methods available in the early 1980s, and the elucidation of the gross structure[8, 16] was first made possible by long-range selective proton decoupling (LSPD) experiments.[18] The absolute stereostructure of **1** was determined by X-ray crystallographic analysis of ptaquiloside tetraacetate.[19] Thus, **1** was shown to be a norsesquiterpene glucoside of the illudane type, a new type of carcinogen. In the 1970s, a large number of indanone-type sesquiterpenes—such as pterosin B (**3**) as characteristic constituents of bracken—were isolated;[20] they are presumably biosynthesized from the illudane-type precursors such as ptaquiloside. Illudane-type sesquiterpenes occur rather rarely in nature. A representative example is illudin-S (**2**), which is isolated as a toxic compound from a bioluminescent mushroom.[21] Whereas **1** reveals potent cytotoxicity, **2** is known to have antitumor activity.



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3.2. Reactivity

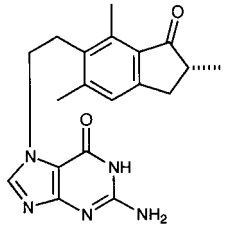
As previously mentioned, ptaquiloside (**1**) is an unstable compound which easily undergoes reaction with acids or bases to give stable aromatic compounds with the indanone skeleton, such as pterosin B (**3**, Scheme 1).[16] In a weakly alkaline aqueous solution (pH 8), **1** was readily transformed into the unstable dienone **4** with concomitant liberation of D-glucose (Scheme 1). Dienone **4** gradually reacted to form **3** under alkaline conditions, whereas it was immediately converted into **3** under weakly acidic conditions. Dienone **4** is a strong electrophile and acts as a powerful alkylating agent toward a variety of nucleophiles such as amino acids. The heteroatoms (oxygen, nitrogen, and sulfur) of amino acids are expected to be alkylated by **4**. Indeed, during the reaction of methionine and **4**, the sulfur atom of the amino acid was selectively alkylated to provide *S*-alkylmethionine **5**. This result indicates that the toxic dienone **4** is effectively converted into the nontoxic indanone compound by a sulfur-containing amino acid. Since indanone compounds such as pterosin B show no carcinogenicity,[20] sulfur-containing amino acids can potentially be used as detoxifying agents for ptaquiloside.

4. DNA Damage by the Ultimate Carcinogen

The covalent binding of carcinogenic alkylating agents with DNA is an important event in the mechanism of action of the agents, and these chemical modifications (damages) of DNA are generally thought to trigger chemical carcinogenesis.[22] Carcinogens are divided into those that react directly with DNA and/or proteins without enzymatic activation, and those that require metabolic activation to produce reactive electrophiles, termed ultimate carcinogens, which react with DNA and/or proteins.[22, 23] The chemical properties of ptaquiloside indicate that it is to be classified as a directly acting carcinogen and that the dienone **4** is the ultimate carcinogen, the activated form of **1**. Considering the fact that most natural carcinogens undergo metabolic activation to lead to ultimate carcinogens, **1** is unique in that it produces the ultimate carcinogen **4** without metabolic activation. In the case of carcinogens that require metabolic activation, it is generally difficult to obtain adequate amounts of the ultimate carcinogens necessary for chemical studies. Therefore, it was advantageous that the amount of the ultimate carcinogen **4** sufficient for bioorganic studies was readily available in the case of the bracken carcinogen **1**.

4.1. DNA Alkylation by the Ultimate Carcinogen[24]

The reaction of salmon sperm DNA with the ultimate carcinogen **4** under physiological conditions (pH 7.5, 37 °C) resulted in the formation of the modified DNA. Thermal hydrolysis of the modified DNA (90 °C, 30 min) produced *N*-7-alkylguanine **6** (1.8 % based on a nucleotide in DNA) and *N*-3-alkyladenine **7** (0.61 %). These results indicated that the thermally unstable sites in DNA were produced by alkylation with **4**.



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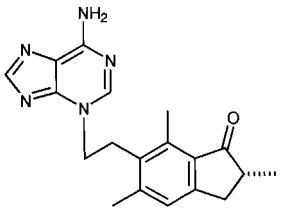

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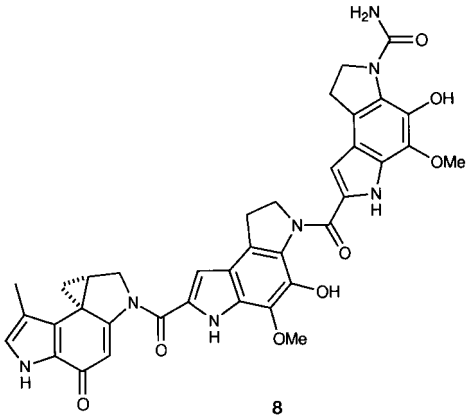
4.2. DNA Cleavage by the Ultimate Carcinogen[24, 25]

The DNA-cleaving activity of **4** was detected by the topological changes in plasmid pBR322 DNA. With the use of 3′- and 5′-^{32}P-labeled DNA fragments, it was shown that **4** cleaved DNA at the adenine and guanine residues. The selectivity of the DNA strand breakage was dependent on incubation time: When a DNA sample was incubated with **4** at 37 °C for 25 h, the DNA cleavage predominantly occurred at the adenine residues, while incubation for 130 h gave additional cuttings at the guanine residues. The key structural feature of the antitumor agent CC-1065 (**8**)[26] is the presence of the cyclopropane ring conjugated with the dienone group. Although the DNA cleavage is postulated to occur by a similar mechanism, there are significant differences between DNA damage by **4** and by **8**.[27] Dieneone **4** forms adducts with guanine residues as well as with adenine residues, while **8** forms adducts only with the adenine residues. Furthermore, **4** causes spontaneous cleavage at the adenine sites under physiological conditions, whereas **8** acts at higher temperatures (> 70 °C).



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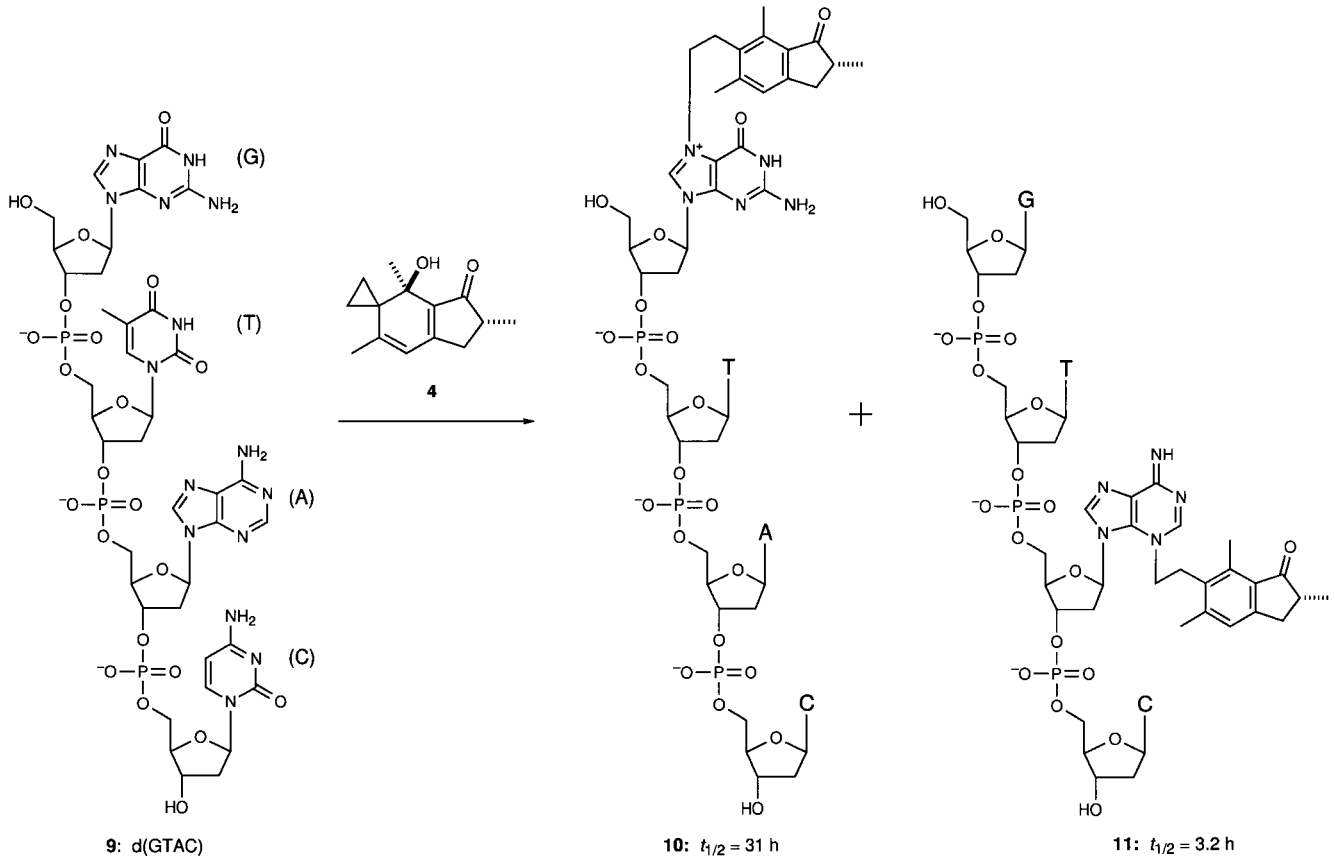
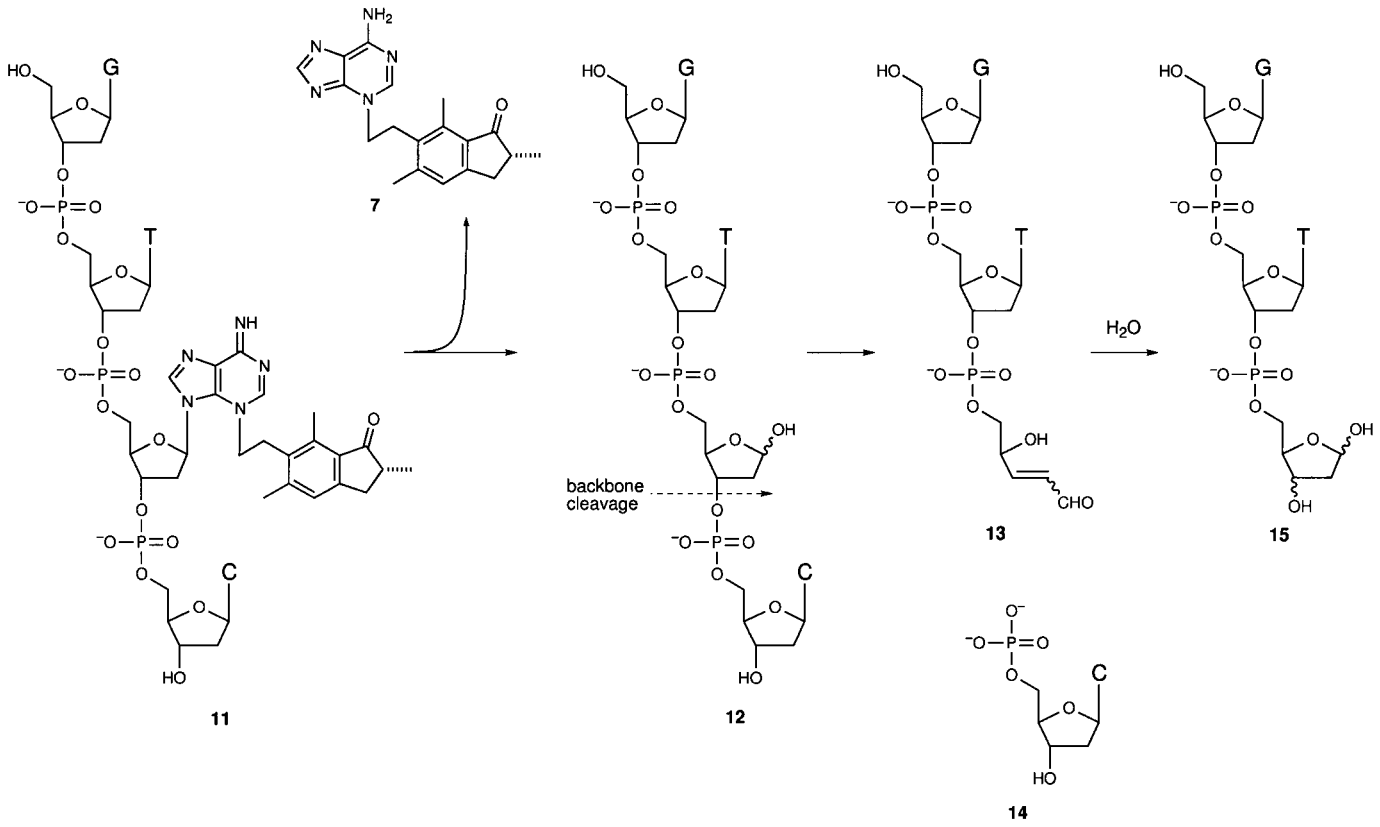
4.3. Reaction of Deoxytetranucleotide d(GTAC) with the Ultimate Carcinogen: Molecular Mechanism of DNA Cleavage[25]

To study the molecular mechanism of DNA cleavage, deoxytetranucleotide d(GTAC) (**9**) was chosen as a model DNA substrate. The reaction of d(GTAC) with the ultimate carcinogen **4** (pH 7.5, 0 °C, 15 h) afforded the two unstable adducts **10** and **11** (Scheme 2). The adduct alkylated at adenine (**11**) was about ten times more unstable than that alkylated at guanine (**10**; $t_{1/2}$ (pH 7.5, 32 °C) = 3.2 and 31 h, respectively).[28] Adduct **11** was transformed into d(GT-deoxyribose-C) (**12**) with liberation of *N*-3-alkyladenine **7** (Scheme 3). Subsequently, the backbone of **12** was cleaved under physiological conditions (pH 7.5, 32 °C, 29 d) to afford deoxycytidine-5′-phosphate **14** and a mixture of aldehydes **13**. The latter was gradually converted into a mixture of alcohols **15** by the Michael addition of water. Thus, the molecular mechanism of the DNA cleavage by **4** can be depicted as



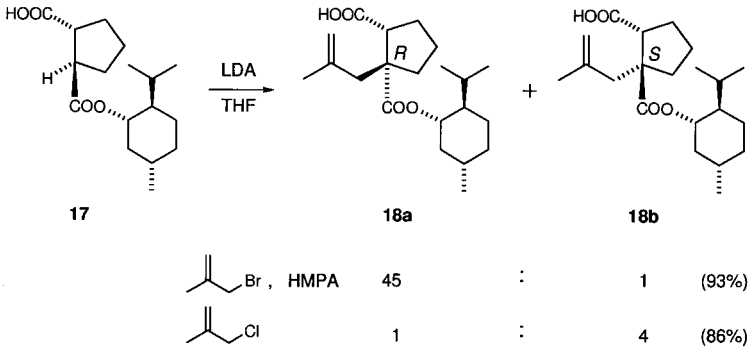
9: d(GTAC) **10**: $t_{1/2}$ = 31 h **11**: $t_{1/2}$ = 3.2 h

Scheme 2. Cleavage of deoxytetranucleotide d(GTAC) (**9**) with the ultimate carcinogen **4**—part 1.



Scheme 3. Cleavage of deoxytetranucleotide d(GTAC) (**9**) with the ultimate carcinogen **4**—part 2.

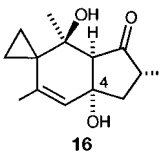
follows: The ultimate carcinogen **4** reacts with DNA to form *N*-3-alkyladenine or *N*-7-alkylguanine adducts. Spontaneous cleavage of the *N*-glycosidic linkage then predominantly takes place at the modified adenine (or guanine) residues to produce abasic sites, where the backbone cleavage gradually occurs by a β-elimination reaction.



Scheme 4. Diastereoselective alkylation of the common chiral precursor **17**.

5. Synthesis of Both Enantiomers of the Ultimate Carcinogen and Their DNA-Cleaving Activities[29]

The challenge of synthesizing ptaquilosin (**16**),[30] the aglycon of the carcinogen ptaquiloside (**1**), combined with the DNA-cleaving activities of the ultimate carcinogen **4** that is produced by alkali treatment of **16**, prompted us to explore the synthesis and properties of both enantiomers of **16**. To efficiently obtain both enantiomers of **16**, the two diastereomers **18a** and **18b** (*R* and *S* configuration, respectively, at the newly constructed quaternary carbon atom) would be stereoselectively prepared from a common chiral precursor **17** (Scheme 4).[31] The lithium enolate generated from (+)-menthyl (1*R*,2*R*)-cyclopentane-1,2-dicarboxylate (**17**) was alkylated with methallyl bromide in the presence of hexamethyl phosphoramide (HMPA) at $-78\,^{\circ}C$ to almost exclusively afford diastereomer **18a** (Scheme 4). On the other hand, the same lithium enolate was alkylated with methallyl chloride in the absence of HMPA at $-20\,^{\circ}C$ to predominantly provide the other diastereomer **18b**, which was produced by alkylation from the sterically more hindered side of the enolate. The plausible intermediary complex for this contrasteric[32] alkylation is shown in Figure 4.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The instability of ptaquilosin poses a multitude of serious problems for its synthesis. The presence of the hydroxyl group at C4, which is liable to be dehydrated, is one of the major reasons for the instability of **16**. Therefore, we devised an approach

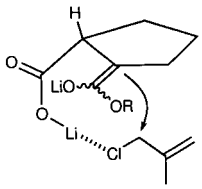


Figure 4. The plausible intermediary complex for the contrasteric alkylation. R = (+)-menthyl.

wherein the angular hydroxyl group is introduced under extremely mild conditions in the very last step. Scheme 5 outlines the synthesis of the natural enantiomer of **16** starting from diastereomer **18a**. The crucial step of the synthesis was deformylative oxidation of aldehyde **27**, which led to **16** via hydroperoxide **28** under sufficiently mild conditions.

The unnatural enantiomer of **16** was synthesized from diastereomer **18b**. With both enantiomers of **16** in hand, we were interested in the problem of whether or not there is a difference in reactivity between the two enantiomers of the ultimate carcinogen **4** toward DNA. The ultimate carcinogen **4** derived from natural ptaquilosin was more efficient (ca. twofold) with respect to DNA cleavage than **4** obtained from unnatural **16**. Other studies directed toward the synthesis of **16** have been reported.[33, 34]

6. Synthesis of Artificial Analogues of the Ultimate Carcinogen

The strong electrophilicity of the ultimate carcinogen **4** is due to the presence of a reactive cyclopropane ring that is conjugated with the keto group and is part of a cyclopropylcarbinol system. The reactivity of the cyclopropane ring in **4** seems to be enhanced by the cooperative action of these two structural factors. From the standpoint of the structure–activity relationship, it was of interest to prepare simple analogues of **4** and to compare their DNA-cleaving activities. Furthermore, it was expected that the simple synthetic analogues would be more stable than the labile ultimate carcinogen **4**. Therfore, we synthesized two simple analogues: ketone **29**, which possesses the cyclopropane ring conjugated with a keto group, and alcohol **30**, which only retains the cyclopropylcarbinol system[35] (Figure 5). Ketone **29** is much more stable than the ultimate carcinogen **4**, whereas alcohol **30** is less stable than **4**. It is remarkable that the artificial analogues **29** and **30** cleaved DNA as effectively as the natural ultimate carcinogen **4**.

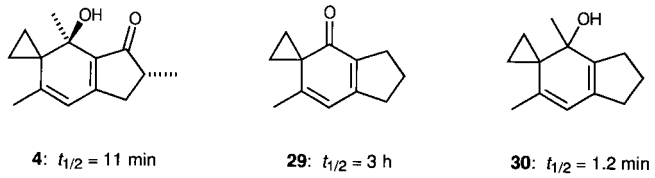
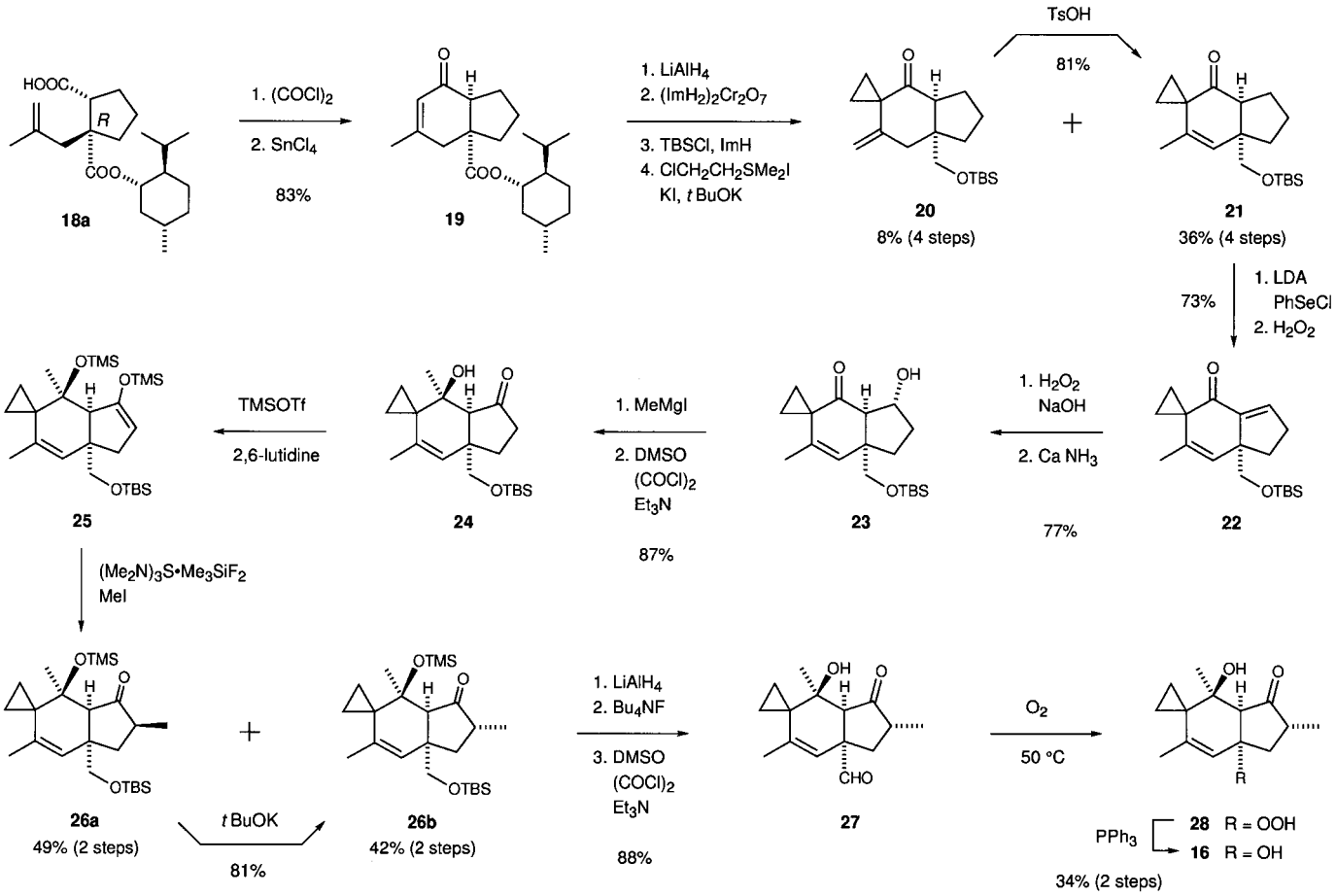


Figure 5. Structures of the ultimate carcinogen **4** and the two artificial analogues **29** and **30**. The half-lives $t_{1/2}$ were measured under physiological conditions (pH 7.5, 26 °C).



Scheme 5. Synthesis of natural ptaquilosin (**16**). TBS = tBuMe$_2$Si, LDA = lithium diisopropylamide, TMS = Me$_3$Si, Tf = F$_3$CSO$_2$.

7. Related Studies on Ptaquiloside

Since we reported the isolation of ptaquiloside from bracken, studies on the qualitative and quantitative analysis of **1** in bracken were performed by a number of groups. For example, Smith et al. reported the presence of **1** in rock fern (*Cheilanthes sieberi*) in New Zealand and Australia,[36] and Alonso-Amelot et al. described that bracken fern collected in the tropical Andes of Venezuela contained **1**.[37] Analyses of **1** in bracken fern from England[38] as well as Costa Rican and Canadian fern[39] were performed. Natori et al. revealed that while **1** does not show mutagenicity under the standard conditions of the Ames test, the dienone **4** does. Thus, they developed a convenient method for the detection of **1** with a modified Ames test.[40, 41] Natori et al. investigated the mutagenic constituents in ferns of the *Pteridaceae* family[42] and isolated mutagenic compounds such as hypolosides A (**31**), B (**32**), and C (**33**)[43] (Figure 6) as well as dennstoside A[44] and determined their structures, which are similar to that of **1**.

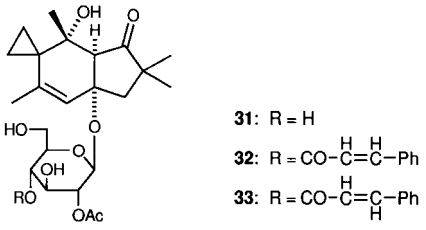


Figure 6. Structures of hypolosides A (**31**), B (**32**), and C (**33**).

Several groups have pointed out the possible human hazard of a bracken carcinogen, especially the transfer of a bracken carcinogen to milk. There were reports that milk from cows fed bracken is carcinogenic to mice and rats, which led to the suspicion that milk contains a bracken carcinogen.[45–48] Recently, Alonso-Amelot, Smith et al. revealed that **1** is present in milk from cows fed bracken.[49]

8. Summary and Outlook

In this review the results of studies on a long-sought carcinogen of a bracken fern are described. A carcinogenicity-guided, mild method for extraction was developed, which enabled us to isolate the unstable carcinogen ptaquiloside (**1**). Ptaquiloside was shown to be responsible for a variety of biological activities including carcinogenicity. The structure of **1** was determined to be a norsesquiterpene glucoside of the illudane type. Under weakly alkaline conditions, **1** transformed into the highly reactive ultimate carcinogen **4**, which alkylates the sulfur atom of sulfur-containing amino acids; this finding provides the possibility of using sulfur-containing amino acids as detoxifying agents for the carcinogen **1**. The ultimate carcinogen **4** not only alkylates the adenine and guanine sites of DNA, but also cleaves DNA; the molecular mechanism of this DNA cleavage was disclosed in detail. Both enantiomers of ptaquilosin (**16**), the aglycon of **1**, were synthesized, and the DNA-cleaving activities of the natural ultimate carcinogen **4** were shown to be stronger than those of unnatural **4**. Two artificial analogues **29** and **30**, which have simpler structures than the ultimate carcinogen **4**, were prepared. Their DNA-cleaving activities proved to be comparable to those of natural **4**. Since **1** is a potent and unique carcinogen that can now be obtained from the plant in large amounts, there will be opportunities in the future for this compound to be used for investigating the detailed mechanism of chemical carcinogenesis.[50]

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