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Antiproliferative Activity and Effect on $GABA_A$ Receptors of Callitrisic Acid Derivatives

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Supporting information Synthetic procedures and copies of ^{1}H NMR and ^{13}C NMR spectra for key synthesized compounds are available online at http://www.thieme-connect.de/products

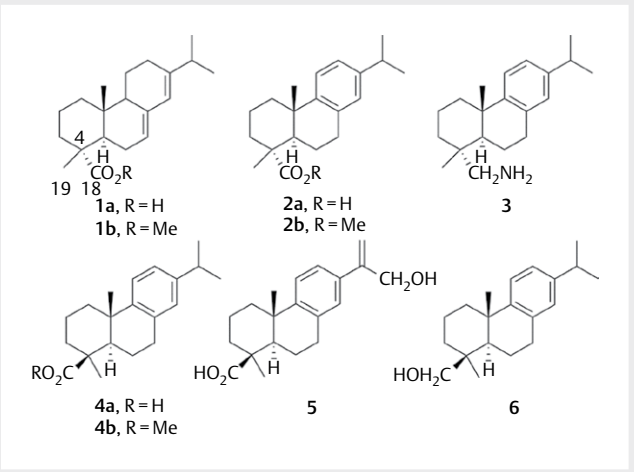
ABSTRACT

The semisynthesis and biological activity of the naturally occurring abietane diterpenoids callitrisic acid (**4a**; 4-epidehydroabietic acid) and callitrisinol (**6**) are reported. These compounds and jiadifenoic acid C (**5**) were obtained from methyl callitrisate (**4b**) isolated from Sandarac resin for biological evaluation and comparison with the biological activities of C4 epimers such as dehydroabietic acid (**2a**). In particular, the antiproliferative activity against a panel of six representative human solid tumor cell lines (A549, HBL-100, HeLa, SW1573, T-47D, WiDr) and the effect on $GABA_A$ receptors ($\alpha_1\beta_2\gamma_{2s}$) were evaluated. The GI_{50} values were in the range of 3.4–61 µM and the potentiation of I_{GABA} was 269–311 % at 100 µM. Callitrisinol (**6**) was found to be 6.7-fold more potent than the reference etoposide in the WiDr (colon) cancer cell line. The role of the stereogenic center at C4 for antiproliferative and $GABA_A$ receptor modulating activities in the dehydroabietane scaffold has thus been revealed.

Introduction

At present, about fifty percent of commercial pharmaceutical drugs are derived from natural sources [1]. The abietane-type and related diterpenoids are a class of naturally occurring terpenoids in the plant kingdom, which have demonstrated a wide range of biological activities against cancer and a variety of infectious diseases (viral and bacterial) [2]. Several research groups have explored the potential as chemotherapeutic agents of abietanes by means of semisynthetic derivatives from abietic acid (**1a**)-derived materials such as dehydroabietic acid (**2a**, DHA) and dehydroabietylamine (**3**), also called leelamine (▶ **Fig. 1**) [3]. For example, DHA displays not only antiulcer and antimicrobial properties, but also antitumor effects [2, 3]. Recently, DHA was reported as a positive $GABA_A$ receptor modulator inducing significant receptor modulation in the oocyte assay, with a maximal potentiation of I_{GABA} of 682.3 ± 44.7 % at 100 µM [4].

DHA displays an equatorial carboxylic group located at C18, while in other natural congeners, the carboxylic group adopts the axial configuration (C19) as in 4-epidehydroabietic acid or callitrisic acid (**4a**; ▶ **Fig. 1**). Callitrisic acid (**4a**) is a diterpenoid acid contained in the resins of several Callitris species, a small genus of the family Cupressaceae, mostly found in Australia though also present



▶ **Fig. 1** Common abietane starting materials and tested compounds.

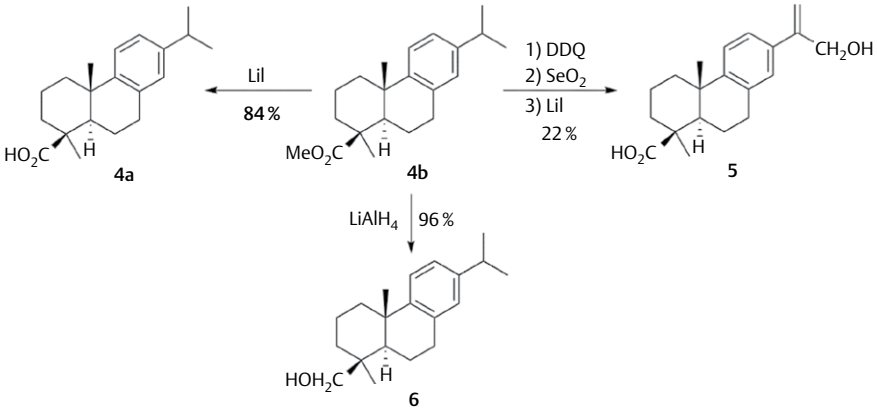
in North Africa [5]. It was simultaneously reported in the late 1960s as a new natural product from the resins of Callitris species (Australian sandarac resin) by Gough [6], and from the Australian white cypress pine Callitris columellaris F. Muell. by Carman and Deeth [7]. This acid also occurs in plants of the genera Juniperus, Calceolaria, and Illicium [2]. Its biological properties have not been studied in depth, especially those of their derivatives, due to limited availability of the parent acid. Recently, a series of related acids having a C19 carboxylic group have been isolated, such as jiadifenoic acids A-I [8]. These, including callitrisic acid, have shown important antiviral properties against Coxsackie virus. We have recently developed the synthesis of jiadifenoic acid C (**5**; ▶ **Fig. 1**) from methyl callitrisate (**4b**) isolated from Moroccan sandarac resin [9]. The ready availability of these materials from our studies (**4b-5**) and the absence of their biological studies as well as chemical manipulation prompted us to carry out this research. Thus, in continuation of our research program to discover bioactive terpenoids [10–13], we carried out the synthesis of several derivatives of methyl callitrisate (**4b**), including callitrisic acid (**4a**), jiadifenoic acid C (**5**), and 4-epidehydroabietol or callitrisinol (**6**) together with an evaluation of their antiproliferative and modulating $GABA_A$ receptor activities. Herein, the evaluation of compounds **1**-**6** against a panel of six representative human solid tumor cell lines and their effect on $GABA_A$ receptors ($\alpha_1\beta_2\gamma_{2s}$) are reported. One aim was the study of the influence of the C4 stereochemistry in the biological activities. This work can also help the design and synthesis of novel abietane-based bioactive compounds.

Results and Discussion

The compounds were synthesized from methyl callitrisate (**4b**), which was obtained from commercially available sandarac resin, following our reported protocol [9] as outlined in ▶ **Fig. 2**. The synthesis of callitrisic acid (**4a**) and 4-epidehydroabietol or callitrisinol (**6**) was straightforward using functional group manipulation of methyl callitrisate (**4b**). Thus, nucleophilic methyl ester cleavage of **4b** with LiI afforded callitrisic acid (**4a**) in an 84 % yield, while reduction with $LiAlH_4$ gave callitrisinol (**6**) in a 96 % yield (▶ **Fig. 2**). Jiadifenoic acid C (**5**) was prepared following our method [9] by regioselective dehydrogenation of methyl callitrisate (**4b**) with 2,3-dichloro-5,6-dicyanoquinone (DDQ), followed by allylic oxidation with catalytic selenium dioxide and tert-butyl hydroperoxide (TBHP) as a co-oxidant and, finally, methyl ester cleavage with LiI to afford jiadifenoic acid C (**5**) in a 22 % overall yield (▶ **Fig. 2**).

With compounds **4**-**6** in hand, the antiproliferative activity against six representative human solid tumor cell lines, A549 (lung), HBL-100 (breast), HeLa (cervix), SW1573 (lung), T-47D (breast), and WiDr (colon), was studied using the sulforhodamine B (SRB) assay [14]. The results expressed as GI_{50} are given in ▶ **Table 1**. The standard anticancer drugs etoposide and cisplatin were used for comparison. All compounds were active ($GI_{50} < 100$ µM) in the cell lines tested, with jiadifenoic acid C (**5**) being the least potent compound with GI_{50} values in the range of 19–61 µM against all cell lines, while compounds **4b** and **6** were the most potent at a similar level. In particular, compound **6** was the most potent against WiDr cells ($GI_{50} = 3.4$ µM), while compound **4b** was the most potent against T-47D cells ($GI_{50} = 8.8$ µM), being 6.7- and 2.5-fold more potent than the reference compound etoposide, respectively. In general, the order of activity in the callitrisic series with different functional groups at C19 was alcohol ≥ ester > acid. Our previous study on the biological activity of dehydroabietic acid (**2a**) derivatives (HeLa and Jurkat cell lines) was also consistent with this order of activity [15]. Though the GI_{50} values obtained with the SRB assay and IC_{50} values obtained with the MTT assay are not fully comparable, it is worth to note that the dehydroabietic acid derivatives (C18-functionalized) in our previous study [15] gave IC_{50} values in the range of 45–337 µM for HeLa cells. This fact involved less active compounds in the dehydroabietic series. In order to confirm this activity tendency, the GI_{50} values for dehydroabietic acid (**2a**) and its methyl ester (**2b**) in A549, HBL-100, HeLa, SW1573, T-47D, and WiDr cells were obtained and compared with the values of the semisynthetic callitrisic acid (**4a**) series. Thus, in general, it can be concluded that our C19-functionalized callitrisic series (**4a**, **4b**) was more potent than the corresponding C18-functionalized series. Also, a SAR trend is that a C19 hydroxymethyl group produced the best antiproliferative activity, whereas a C19 carboxylic group led to less active compounds, including jiadifenoic acid C (**5**) as the least potent. Thus, the presence of an allylic alcohol at C13 seems to be detrimental for the antiproliferative activity.

In another experiment based on the oocyte assay, compounds **4**-**6** were tested for their effects on $GABA_A$ receptors ($\alpha_1\beta_2\gamma_{2s}$) by means of the two-microelectrode voltage clamp technique in Xenopus laevis oocytes [4]. All compounds were screened at concentrations of 10 and 100 µM and compared with DHA (**2a**) as a positive control [4]. The results are summarized in ▶ **Table 2**. Callitrisinol (**6**) modulated I_{GABA} at 10 and 100 µM (potentiation of I_{GABA} of 116 % and 311 %, respectively), while methyl callitrisate (**4b**) and jiadifenoic acid C (**5**) were inactive. Callitrisic acid (**4a**) only enhanced GABA evoked currents at 100 µM (potentiation of I_{GABA} of 269 %). Thus, it can be concluded that the presence of an allylic alcohol at C13 significantly reduced the $GABA_A$ receptor modulating effect, while the presence of a C19 hydroxymethyl group increases activity. On comparing $GABA_A$ modulating activity of DHA (**2a**; potentiation of I_{GABA} of 789 % at 100 µM) with its C4-epimer, callitrisic



▶ **Fig. 2** Synthesis of tested compounds **4a**, **5**, and **6** from methyl callitrisate (**4b**).

▶ **Table 1** Antiproliferative activity (GI_{50}) of callitrisic and dehydroabietic acid derivatives **1**–**6** against human solid tumor cells[a].

	Cell line (origin)					
Compound	**A549 (lung)**	**HBL-100 (breast)**	**HeLa (cervix)**	**SW1573 (lung)**	**T-47D (breast)**	**WiDr (colon)**
1a	24.0 ± 2.1	37 ± 3.4	33.0 ± 4.9	62.0 ± 1.8	>100	95.0 ± 6.4
1b	17.0 ± 2.0	17.0 ± 0.3	15.0 ± 1.8	16.0 ± 5.0	14.0 ± 4.9	13.0 ± 3.6
2a	25.0 ± 5.2	39.0 ± 7.3	25.0 ± 9.1	47.0 ± 1.7	40.0 ± 5.9	26.0 ± 8.4
2b	15.0 ± 2.6	19.0 ± 0.2	15.0 ± 3.0	22.0 ± 6.2	16.0 ± 4.7	10.0 ± 3.6
4a	16.0 ± 3.4	36.0 ± 9.9	15.0 ± 6.4	32.0 ± 6.2	33.0 ± 1.0	31.0 ± 7.1
4b	10.0 ± 1.9	14.0 ± 5.0	16.0 ± 2.5	17.0 ± 2.2	8.8 ± 2.7	6.4 ± 2.1
5	19.0 ± 7.2	61.0 ± 8.7	27.0 ± 4.0	55.0 ± 4.3	37.0 ± 5.4	47.0 ± 1.6
6	11.0 ± 5.3	17.0 ± 1.9	13.0 ± 0.6	18.0 ± 1.8	10.0 ± 1.6	3.4 ± 1.3
Etoposide	1.5 ± 0.3	1.4 ± 0.1	3.3 ± 1.6	15.0 ± 1.5	22.0 ± 5.5	23.0 ± 3.1
Cisplatin	4.9 ± 0.2	1.9 ± 0.2	1.8 ± 0.5	2.7 ± 0.4	17.0 ± 3.3	23.0 ± 4.3

[a]Values are given in µM and represent the mean ± standard deviation of at least two independent experiments

▶ **Table 2** Potentiation of I_{GABA} in $\alpha_1\beta_2\gamma_{2s}$ receptors by compounds **2a** and **4**–**6**.

Compound	Potentiation of I_{GABA} (%) at 10 µM	Potentiation of I_{GABA} (%) at 100 µM
2a	192	789
4a	14	269
4b	N.A.	N.A.
5	N.A.	N.A.
6	116	311

N.A.: not active

acid (**4a**; potentiation of I_{GABA} of 269 % at 100 µM), it can be concluded that the stereochemistry at C4 is very important for $GABA_A$ activity, with the callitrisic acid series being the least active.

In summary, the compounds in this communication support the importance of the aromatic abietanes with a dehydroabietane skeleton for antiproliferative activity. Therefore, these abietane compounds may be useful leads for the development of novel antitumor drugs. The role of the stereogenic center at C4 for antiproliferative and $GABA_A$ receptor modulating activities in the dehydroabietane scaffold have been revealed. Further studies to identify more structure-activity relationships and enhance the observed activities are under way.

Materials and Methods

General experimental procedures

Optical rotations were measured using a 5-cm cell in a Schmidt-Haensch Polartronic-D polarimeter. NMR spectra were recorded on a 300 MHz spectrometer. All spectra were recorded in $CDCl_3$ as the solvent unless otherwise stated. Complete assignments of ^{13}C NMR multiplicities were made on the basis of DEPT experiments. J values are given in Hz. MS data were acquired on a QTOF spectrometer. Reactions were monitored by TLC using Merck silica gel 60 F-254 in 0.25-mm thick plates. Compounds on TLC plates were detected under UV light at 254 nm and visualized by immersion in a 10 % sulfuric acid solution and heating with a heat gun. Purifications were performed by flash chromatography on Merck silica gel (230–400 mesh). Commercial reagent grade solvents and chemicals were used as purchased unless otherwise noted. Combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure.

Compounds

Compounds **1**, **2**, **4b**, and **5** were prepared according to known procedures [9, 15]. Compound **4b** was used as starting material for the preparation of compounds **4a** and **6**. All compounds prepared in this work exhibit spectroscopic data in agreement with the proposed structures. The purity of all final compounds was 95 % or higher.

Antiproliferative assay

Cells were inoculated onto 96-well microtiter plates in a volume of 100 µL per well at densities of 2500 (A549, HBL-100, and HeLa) and 5000 (SW1573, T-47D, and WiDr) cells per well, based on their doubling times. Compounds **1**-**6** were initially dissolved in DMSO at 400 times the final maximum test concentration. Control cells were exposed to an equivalent concentration of DMSO (0.25 % v/v, negative control). Each compound was tested in triplicate at different dilutions in the range of 0.001–100 µM. The drug treatment started on day 1 after plating. Drug incubation times were 48 h, after which cells were precipitated with 25 µL of ice-cold TCA (50 % w/v) and fixed for 60 min at 4 °C. Then, the SRB assay was performed as previously described [14]. The optical density (OD) of each well was measured at 530 nm using BioTek's PowerWave XS absorbance microplate reader. Values were corrected for background OD from wells containing only medium. The antiproliferative activity for each compound, expressed as GI_{50} values, was calculated according to NCI formulas [14].

Expression of GABAA receptors in Xenopus laevis oocytes

The experiments were carried out following procedures formerly described with a few modifications [4, 16]. Briefly, follicle membranes covering oocytes were enzymatically digested with 2 mg · mL^{-1} collagenase (type 1A). The coding regions of plasmids were sequenced before experimental use. After cDNA linearization, capped cRNA transcripts were produced using the mMESSAGE mMACHINE® T7 transcription kit (Life Technologies). Capped transcripts were polyadenylated using yeast poly(A)polymerase, diluted in nuclease-free water, and stored before injection at -80 °C.

One day after isolation, the oocytes were injected with about 10–50 nL of nuclease-free water containing the different rat cRNAs (100–2000 ng · $µL^{-1}$ per subunit). For expression of wild-type α1β2γ2S, cRNAs were mixed in a ratio of 1:1:10. Electrophysiological experiments were performed using the two-microelectrode voltage clamp technique at a holding potential of − 70 mV, making use of a TURBO TEC 01C amplifier (NPI Electronic) and an Axon Digidata 1440A interface (Molecular Devices). Data acquisition was carried out using pCLAMP v.9.2 (Molecular Devices). The bath solution contained 90 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 5 mM HEPES (adjusted to pH 7.4 using 1 M NaOH). Microelectrodes were filled with 2 M KCl and had resistances between 1 and 3 MΩ.

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Conflicts of Interest

The authors declare no conflict of interest.

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