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Graphical Abstract

A new approach for the total synthesis of spilanthol and analogue with improved anesthetic activity.

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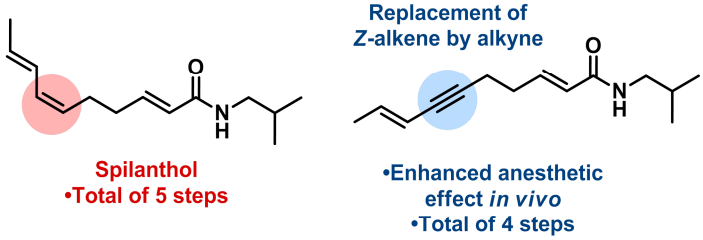

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ABSTRACT

A 5-step synthesis of spilanthol (affinin) is reported, where the route features complete control of alkene geometry during the assembling of the double bonds, with the use of a Sonogashira cross-coupling reaction, a *Z*-selective alkyne semi-reduction and a HWE olefination reaction as the key steps. A simplified analogue was also prepared in 4 steps. Both compounds were found to permeate dermatomed pig ear skin through an *in vitro* Franz-type diffusion cell. The simplified analogue presented a superior anesthetic effect *in vivo*, using the tail flick model, when compared to spilanthol and to the commercial standard EMLA®. These results suggest that both spilanthol and its analogue could be useful as a topical anesthetic in clinical practice.

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1. Introduction

Novel chemical structures can be isolated from natural sources, and they have been applied for drug discovery over the past decades with great success. Indeed, between 1981 and 2014, natural products and molecules inspired by their scaffolds represent more than half of all approved small-molecule drugs.[1]

Spilanthol, also known as affinin (**Figure 1**), is a member of the *N*-alkylamide family and has been found in several natural sources, such as *Acmella oleracea* (jambu), *A. ciliate*, *A. oppositifolia*, *A. radicans*, *Welelia parviceps* and *Heliopsis longipes*.[2,3] Spilanthol is a high-value added compound and is commercially available, however, it is sold with approximately 80% isomeric purity at prices as high as $150.00 for 1 mg or $1,400.00 for 10 mg.[4]

Extracts containing spilanthol, or even the aerial parts of jambu, have been used for centuries in traditional folk medicine and cuisine throughout the world.[5] Thus, several studies performed with spilanthol, or most commonly with enriched plant extracts, exhibited an array of biological activities, including antipyretic,[6] diuretic,[7,8] antimalarial,[9] aphrodisiac,[10,11] anti-inflammatory[12,13] and interestingly, analgesic and anesthetic activity, among many others.[5,13-14]

Local anesthetics, one of the most important advancements in medicine, are chemical substances that cause a reversible loss of sensation due to a blockade of nerve conduction around the site of application.[16] When topically applied, anesthetics produce a superficial loss of sensation in the conjunctiva, mucous membranes or skin and, therefore, are used in numerous medical specialties, such as dentistry, otorhinolaryngology, ophthalmology, urology, aesthetic surgery, among others, to minimize the anxiety, pain or discomfort caused by some procedures.[16,17]

The most widely used topical anesthetics are lidocaine, benzocaine and prilocaine in form of gels, creams, sprays, solutions or ointments. EMLA® (Eutectic Mixture of Local Anesthetics), which is composed of 2.5% of lidocaine and 2.5% of prilocaine (**Figure 1**), is often regarded as the gold standard for topical anesthesia.[18]

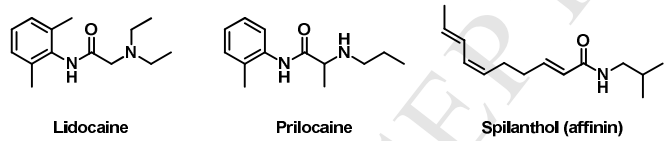

Fig. 1. Chemical structures of lidocaine, prilocaine and spilanthol.

Despite being generally regarded as safe, the use of topical anesthetics requires caution. When the recommended dose is exceeded, systemic effects may occur, with the patient presenting symptoms such as dizziness, respiratory distress, loss of consciousness and even cardiac arrest.[19] Moreover, there are reports of allergic reactions, neurotoxicity, and cardiotoxicity that led some patients to death.[20-22]

In this scenario, the investigation of safer topical anesthetics is highly desired and timely. As far as spilanthol is concerned, it is considered safe by the European Food Safety Authority (EFSA) and jambu oleoresin is classified as a Generally Recognized as Safe (GRAS) substance by the Flavor and Extract Manufacturers Association (FEMA), being included in several alimentary products.[23-24]

In our previous work, we found that ethanolic extracts of aerial parts of *Acmella oleracea* exhibited significant anesthetic activity, similar to the EMLA® effect.[25,26] Since extracts usually present a great variety of compounds with fluctuating contents thereof, depending on the extraction procedure or seasonal factors, we envisioned further exploring the anesthetic activity with spilanthol obtained by chemical synthesis.

Herein, we report a new synthetic approach to obtain spilanthol in high purity, as well as a simplified analogue. The permeation profiles of both compounds were evaluated through *in vitro* experiments with a Franz-type diffusion cell, and their anesthetic effect was assayed *in vivo* using the tail flick model.

2. Results and discussion

2.1. Chemical synthesis

Since its first isolation by Gerber in 1903,[27] four total syntheses of spilanthol have been reported to date, and these works are summarized in **Figure 2A**. The first successful synthesis was reported by Crombie and co-workers in 1963,[28] allowing for the structural elucidation regarding the double bonds stereochemistry for this natural product.

Yamamoto and co-workers approached the synthesis using a convergent strategy with the addition of allyltitanium species over an aldehyde as a key step. Spilanthol was obtained in 88% purity determined by GC-FID analysis.[29,30]

In 1998, Lu and co-workers reported the shortest synthesis of spilanthol, where the key transformation employed was a cotrimerization of acrolein with two equivalents of acetylene, which assembled the conjugated diene fragment with the required geometry in 43% yield. However, the use of acetylene is a drawback of this synthesis, since this gas is extremely flammable and potentially explosive, posing some safety concerns on scale.

From 2009 to 2012, Takasago International Corporation has filed several patents for the synthesis and use of spilanthol.[31-35] The synthesis was developed on multi-gram scale, producing 67.0 g of spilanthol from a single batch, and only extraction, distillation and crystallization were required for the purification steps. However, a poor control over the double bond formation was observed: spilanthol was obtained with 78% for the desired isomer, 18.0% for the all-*E* isomer, and 4% for the (2*E*,6*Z*,8*Z*)-isomer.

Bearing these syntheses and their limitations in mind, we sought to develop a short synthesis of spilanthol and some simple analogues which would not require the use of extremely toxic or dangerous chemicals, and could provide spilanthol with good control of alkene geometry. Thus, we proposed the retrosynthesis depicted in **Figure 2B**. Initially, the C2-C3 double bond would be forged by a Horner-Wadsworth-Emmons (HWE) olefination. The product of this reaction is usually easier to purify when compared to a Wittig reaction, the choice of Yamamoto and Lu, which produces triphenylphosphine oxide and can require multiple purifications.[36]

The C6-C7 olefin could arise from a selective semi-reduction of alkyne **2**, which in turn could be generated *via* a Sonogashira cross-coupling reaction between alkyne **5** and 1-bromo-1-propene as a mixture of isomers (*E*/*Z* 60:40), which is nearly 20 times cheaper than the (*E*)-1-bromo-1-propene. The production of the desired *E*-isomer of compound **2** relied on previous studies which demonstrated that (*E*)-1-bromo-1-propene reacts faster in Sonogashira reactions than the *Z*-isomer,[37] and that double bond isomerization can occur under certain reaction conditions.[38,39]

Synthesis of fragment **3** was planned to be conducted with a direct amidation of the corresponding ethyl ester with isobutylamine.

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Analogue **8** would be prepared *via* a similar route, except for the absence of alkyne semi-reduction (**Figure 2C**). The alkyne was thought to give more rigidity to the molecule, which could impact its bioactivity and stability profile.

Entry [a]	Deviation from the above	*E*:*Z* Ratio [b]	Yield (%) [c]
1	None	91:9	56
2	100 °C	81:19	44
3	100 °C, K_2CO_3 as base	42:58	77
4	100 °C, Et_3N as base	77:23	64
5	60 °C	93:7	45
6	60 °C, Cs_2CO_3 as base	60:40	64
7	60 °C, K_2CO_3 as base	57:43	55
8	60 °C, Et_3N as base	88:12	39
9	60 °C, Piperidine as base	54:46	98

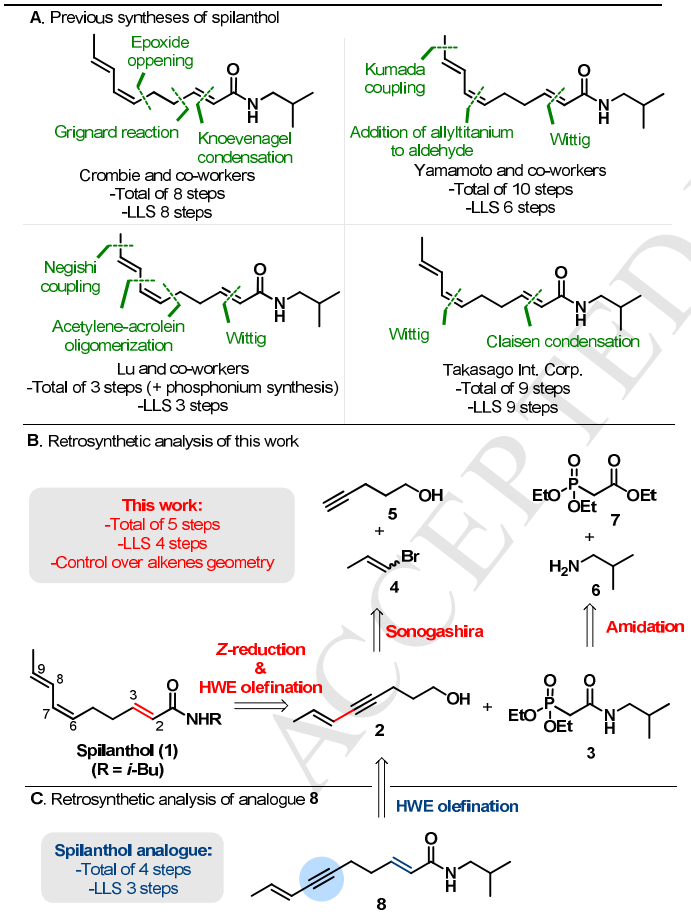

Fig. 2. (**A**) Previous syntheses of spilanthol; (**B**) retrosynthesis of spilanthol and (**C**) analogue **8** in this work.

Initially, we explored the synthesis of (2-(isobutylamino)-2-oxoethyl)phosphonate fragment **3** (**Scheme 1A**). This scaffold has been synthesized by different methods which required multiple steps, the use of toxic reagents or furnished moderate yields.[40-44] We envisioned that a methodology described by Morimoto, Ohshima and co-workers could be beneficial to synthesize this phosphonoacetamide.[45] The reaction was carried out in the absence of solvent, with a small excess of isobutylamine, in the presence of catalytic amounts of lanthanum(III) triflate at room temperature. Phosphonoacetamide **3** was obtained in 93% yield and no side-products were observed, which facilitated its purification.

Next, we turned our attention to the conjugated diene fragment (**Scheme 1B**). The carbon skeleton was constructed using a Sonogashira cross-coupling reaction between 4-pentyn-1-ol and 1-bromo-1-propene as a mixture (*E*/*Z* 60:40).[46]

Our first set of optimizations for the Sonogashira reaction used vinyl bromide **4** as the limiting reactant and excess of alkyne **5**. In an attempt to favor double bond isomerization, experiments performed at higher temperatures (i.e. 100 °C) provided low selectivities and yields (**Table 1**, entries 2-4). When the reaction was performed at 60 °C (**Table 1**, entries 5-9) higher selectivities were obtained, although the yields were low for most of the bases investigated. Curiously, the use of piperidine as base furnished the product in almost quantitative yield (entry 9), but with marginal isomerization favoring the undesired isomer, while the use of diisopropylamine (DIPA, entry 5) led to the highest selectivity, with a moderate yield. As vinyl bromide **4** is a volatile compound (b.p. 58-63 °C), we also attempted the reaction at room temperature (entry 1), which provided a similar result to the test at 60 °C. Due to easier experimental setup, it was chosen as the best condition at this point and the reaction was further investigated (**Table 2**).

Table 1. Optimization of the key Sonogashira reaction.

[a] Scale: 1 mmol of 1-bromo-1-propene (*E*/*Z* 60:40), using a sealed tube, up to 15 h. [b] Determined by ^{1}H NMR analysis of the crude. [c] Isolated yield for duplicates.

For the second round of optimizations, alkyne **5** was employed as the limiting reactant without significant change in yield and selectivity (**Table 2**, entry 1). Triphenylphosphine was found as an important additive to further increase the selectivity towards the *E*-alkene **2**; this reagent decreased the formation of dark solid, which was attributed to palladium black, and the next modifications included the presence of PPh_3. Also, we observed an increase in the reaction kinetics as the reaction went to completion in just 2 hours.

From the evaluated solvents, DMF favored a higher selectivity, ranging from 94:6 to >95:5 with moderate yield (entries 2-4). Use of $Pd(PPh_3)_2Cl_2$ instead of $Pd(PPh_3)_4$ or decreasing the palladium catalyst loading led to smaller selectivities or yields (entries 5-7). Notably, entry 3 presents higher yield, but was not chosen during the spilanthol synthesis because the minor isomer could not be separated by standard techniques, as pointed out previously. Although the yield could not be improved beyond 53%, DIPA was the base of choice since it furnished the best compromise between yield and diastereoselectivity of the product. Under these best conditions, the desired 6*E*-isomer of **2** was obtained in 53% yield in more than 95:5 diastereomeric ratio, as determined by 1H NMR analysis.

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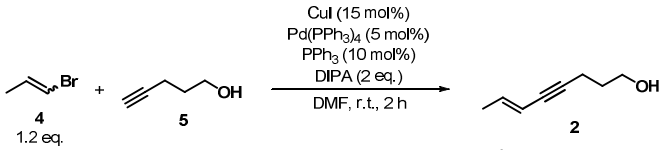
Table 2. Further optimization of the key Sonogashira reaction[a]



[a] Scale: 1 mmol of 4-pentyn-1-ol unless noted otherwise. [b] Determined by ^{1}H NMR analysis of the crude. [c] Isolated yield for duplicates. [d] Scale: 1 mmol to 10 mmol of 4-pentyn-1-ol; lower selectivities were obtained on larger scales. [e] 15 h.

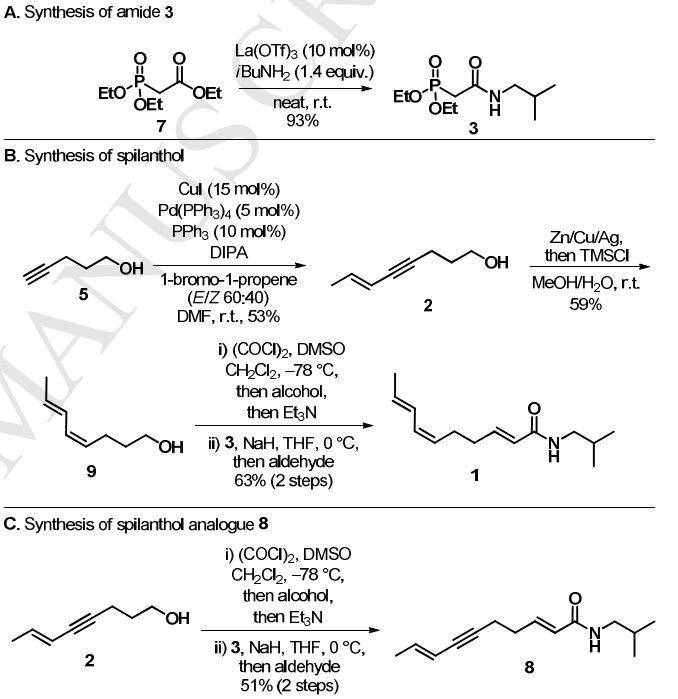
Entry [a]	Deviation from the above	*E*:*Z* Ratio [b]	Yield (%) [c]
1	No PPh_3	91:9	56
2[d]	None	93:7 to > 95:5	53
3	THF as solvent	79:21	77
4	MeCN as solvent	84:16	38
5	$Pd(PPh_3)_2Cl_2$ as Pd source	83:17	49
6[e]	2.5 mol% of $Pd(PPh_3)_4$	>95:5	20
7[e]	1.0 mol% of $Pd(PPh_3)_4$	78:22	11

The diastereoselectivity obtained for the Sonogashira cross-coupling reaction was confirmed by the use of pure (*E*)-1-bromo-1-propene (obtained after isomerization of the *E*/*Z* mixture under basic conditions)[39] and (*Z*)-1-bromo-1-propene (obtained from commercial sources). Thus, employing piperidine as base in DMF at room temperature, the Sonogashira adducts (*E*)- and (*Z*)-**2** were obtained in 81% and 68%, respectively, without any noticeable double bond isomerization (>95:5 d.r. according to ^{1}H NMR analysis). The peaks at 6.04 and 5.90 ppm in the ^{1}H NMR spectra were unequivocally attributed to the (*E*)- and (*Z*)-isomers, respectively, and allowed an easy diastereomeric ratio assessment (see **Figures S7**, **S10**, **S12** and **S14** in the Supporting Information).

The subsequent step to produce the key diene **9** involved a *Z*-selective alkyne semi-reduction with an alloy of zinc, copper and silver in the presence of trimethylsilyl chloride (modified Boland's protocol), based on the report of Hansen.[47] The alkyne reduction proceeded in 59% yield with complete selectivity towards the expected *Z*-isomer (>95:5 by ^{1}H NMR analysis).

Finally, the conclusion of the spilanthol synthesis required the connection of the fragments and generation of a third double bond with *E*-geometry. To accomplish this task, alcohol **9** was subjected to a Swern oxidation, and the crude aldehyde was then employed in a Horner-Wadsworth-Emmons reaction with phosphonoacetamide **3**. The newly created alkene exclusively presented an *E*-geometry (> 95:5 by ^{1}H NMR analysis) and spilanthol was thus obtained in 63% yield, over two steps. Its spectroscopic data (^{1}H and ^{13}C NMR) were identical to those of an authentic sample of spilanthol obtained from a commercial source (see Supporting Information for details).

The synthesis of analogue **8** required a similar sequence (**Scheme 1C**), but no alkyne reduction was necessary. For this final product, the minor isomer constituted by the alkene with 8*Z*-geometry could be removed after standard flash chromatography. On the other hand, the minor 8*Z*-isomer of spilanthol could not be removed by the same procedure, and for this reason, we extensively studied the initial Sonogashira coupling aiming for a higher *E*-selectivity.



Scheme 1. Synthesis of spilanthol and analogue **8**

2.2. Biological evaluation

2.2.1. In vitro permeation studies

Aiming for the application of spilanthol and analogue **8** as topical anesthetics, both compounds were incorporated in the formulation of bioadhesive films (see experimental section for details). The *in vitro* permeability was evaluated using Franz-type vertical diffusion cells, an apparatus commonly used to evaluate the permeation of compounds through the skin or mucosa.[48] As a barrier, dermatomed pig ear skin was used, due to the similarities between the pig tissues and the human skin.[49]

Other research groups have described that spilanthol was able to permeate the skin and mucosa, and could also act as an enhancer, increasing the permeation of some substances through the dermis.[50-52] In our study, spilanthol and analogue **8** showed a similar permeation profile through dermatomed pig ear skin, as can be seen in **Figure 3** and the permeation profile of spilanthol was compatible with those observed in the literature.[51] The flux (spilanthol: 0.78 ± 0.11, analogue **8**: 0.88 ± 0.09 $\mu g.cm^{-2}.h^{-1}$) and

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lag time (spilanthol: 0.66 ± 0.41, analogue **8**: 0.81 ± 0.43 h) for spilanthol and analogue **8** were identical within the limits of experimental error.

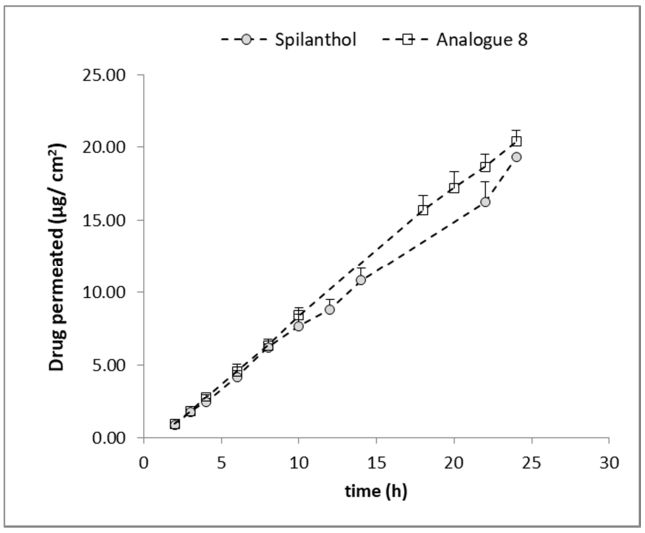

Fig. 3. Permeation profiles of spilanthol and analogue **8** applied under finite dose conditions (mean values ± SEM)

This result can be understood from the similar molecular structures and physical parameters, such as log P and polar surface area which both compounds share. Since spilanthol and analogue **8** exhibited similar permeation profiles, we then evaluated their anesthetic activity.

2.2.2. In vivo anesthetic effect

The tail flick model was employed for the anesthetic evaluation of spilanthol and analogue **8**. This model, first described by D'Amour and Smith,[53] has been used to evaluate the anesthetic effect of topical formulations by several groups.[54-56]

The bioadhesive films containing spilanthol and analogue **8** were prepared according to our previous work using the natural polymer hydroxyethyl cellulose (HEC) and some adjuvants[57] (see experimental section and Supporting Information for more details). Initially, a bioadhesive film composed of hydroxyethyl cellulose, Tween® 80, glycerin and Transcutol®, prepared without an anesthetic compound was evaluated as a negative control, and showed no antinociceptive effect (data not shown in **Figure 4**), which confirmed that the film basis is inactive.

We observed through the tail flick test that spilanthol and EMLA® showed similar anesthesia duration (**Figure 4**), while analogue **8** presented an increased anesthetic effect ($p<0.01$). Taking into account the previous studies with *jambu* extracts, our work confirms that the antinociceptive activity is indeed due to the presence of spilanthol, and not to an association of several chemical components.

In view of the superior activity exhibited by analogue **8**, we hypothesized that the alkyne group in compound **8** imposes conformational constraints on the molecule when compared to spilanthol, which might enhance affinity or selectivity with a pain receptor. Further studies are being conducted by our group in order to elucidate the mode of action of spilanthol and analogues.

Despite the recent advances in topical anesthesia, where a variety of new drug delivery systems have been developed to enhance and improve anesthesia, topical anesthetics remain underused.[58] This scenario is partly due to the long onset of action that most topical anesthetics require, and also due to a lack of effectiveness in some procedures.[59] The results presented here showed that analogue **8** and spilanthol are potent anesthetics with a short onset of action (5 min). Both compounds demonstrated anesthesia similar or superior to EMLA®, the anesthetic currently considered as the gold standard in topical anesthesia. It is important to highlight that the effect observed for spilanthol and its analogue required extremely low doses (approximately 100 µg).

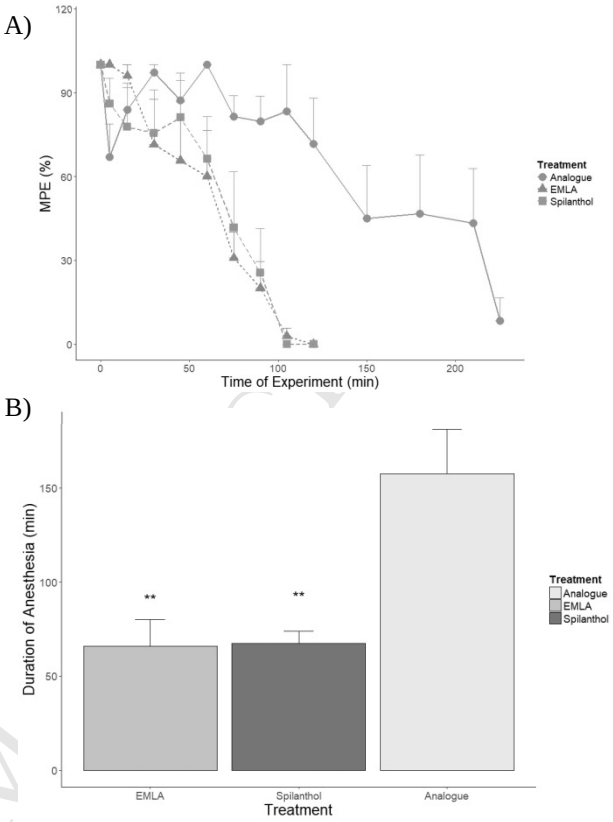

Fig. 4. Effects of topical administration of EMLA®, spilanthol and analogue **8** in the tail flick assay. Time-course (min) showing the percent of animals with anesthesia (A) and the duration of anesthesia (B). (n = 5-6/group). Data expressed as mean±SEM AUC for MPE (%) $p< 0.001$ when spilanthol and EMLA were compared to analogue **8**. Duration of anesthesia: $^{**}p<0.01$. One-way analysis of variance with Tukey posthoc test.

3. Conclusions

This work combined modern synthetic chemistry and bioassays to address the development of anesthetics for dentistry, surgeries, among other applications. We presented a new route for the synthesis of the *N*-alkylamide spilanthol, which features complete control over the geometry of its three double bonds. A simplified analogue was also prepared with an alkyne replacing the *Z*-alkene, which required one step less when compared to spilanthol. Both compounds presented similar profiles for *in vitro* permeation across dermatomed pig ear skin. Their anesthetic profile was evaluated *in vivo* with the tail flick model, and analogue **8** showed a superior effect when compared to spilanthol and the commercial anesthetic EMLA®. These results open the door to exploration of new spilanthol derivatives and further investigations of compound **8** as a potential tool for medicine.

4. Experimental section

4.1. Chemical synthesis

Starting materials and reagents were obtained from commercial sources and used as received unless otherwise specified. Dichloromethane, triethylamine, diisopropylamine (DIPA) were treated with calcium hydride and distilled before use. Tetrahydrofuran (THF) was treated with metallic sodium

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and benzophenone and distilled before use. Dry *N*,*N*-dimethylformamide (DMF) and dimethylsulfoxide (DMSO) were obtained from Aldrich. Anhydrous reactions were carried out with continuous stirring under an atmosphere of dry nitrogen. Progress of the reactions was monitored by thin-layer chromatography (TLC) analysis (Merck, silica gel 60 F254 on aluminum plates), unless otherwise stated. Flash chromatography purifications were performed with silica gel 60, 220-440 mesh, Sigma-Aldrich. ^{1}H NMR, ^{13}C NMR and ^{31}P NMR spectra were recorded on Bruker 250, 400 and 600 MHz spectrometers, the chemical shifts (δ) were reported in parts per million (ppm) relative to deuterated solvent as the internal standard ($CDCl_3$: 7.26 ppm for ^{1}H NMR and 77.0 ppm for ^{13}C NMR), coupling constants (*J*) are in hertz (Hz). The following abbreviations were used to designate chemical shift multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, m = multiplet, br. = broad signal. NMR spectra were processed using ACD/NMR Processor Academic Edition version 12.01. High resolution mass spectra (HRMS) were recorded on a Waters Xevo Q-Tof apparatus operating in positive ion electrospray mode (ESI+). Fourier Transform Infrared (FTIR) spectra were recorded on a Thermo Scientific Nicolet iS5, the principal absorptions are listed in cm^{-1}. IUPAC names of the compounds were generated using ChemBioDraw Ultra 12.0.

*4.1.1. Diethyl (2-(isobutylamino)-2-oxoethyl)phosphonate (**3**)*

A 5 mL round-bottomed flask with a magnetic stirrer was charged with lanthanum (III) triflate hydrate ($La(OTf)_3$•xH_2O, 325 mg, 10 mol%), the flask was heated with a heat-gun over 220 °C for 15 min under vacuum (<1 mbar), and was filled with dry N_2. Next, triethyl phosphonoacetate (**7**, 1.136 g, 4.9 mmol, 1.0 eq.) and isobutylamine (**6**, 507 mg, 6.9 mmol, 1.4 eq.) were added *via* syringe (*no exothermic reaction was observed during addition of all chemicals*). The reaction was stirred for 4 h at room temperature, then the crude mixture was filtered through a column of silica using CH_2Cl_2:MeOH (100:0 to 90:10) as eluent to furnish the amide **3** (1.15 g, 4.6 mmol) as a colorless oil in 93% yield. R_f 0.19 (SiO_2, EtOAc). ^{1}H NMR (250 MHz, $CDCl_3$): δ 0.85 (d, *J* = 6.6 Hz, 6H), 1.27 (t, *J* = 7.1 Hz, 6H), 1.72 (non, *J* = 6.9 Hz, 1H), 2.75 (s, 1H), 2.83 (s, 1H), 3.02 (t, *J* = 6.3 Hz, 2H), 4.07 (dq, *J* = 8.2, 7.1 Hz, 4H), 6.92 (s, 1H). ^{31}P NMR (101 MHz, $CDCl_3$): δ 22.8-23.5 (m, 1P). ^{13}C NMR (62.9 MHz, $CDCl_3$) *δ*: 16.2 (d, $J_{C\text{-}P}$ = 6.4 Hz, CH_3), 19.9 (2xCH_3), 28.2 (CH), 34.8 (d, $J_{C\text{-}P}$ = 131 Hz, CH_2), 47.0 (CH_2), 62.5 (d, $J_{C\text{-}P}$ = 6.9 Hz, 2CH_2), 165.8 (d, $J_{C\text{-}P}$ = 3.2 Hz, C).

*4.1.2. (E)-oct-6-en-4-yn-1-ol (**2**)*

A round-bottomed flask was charged with PPh_3 (347 mg, 1.3 mmol, 10 mol%), CuI (382 mg, 2.0 mmol, 15 mol%) and $Pd(PPh_3)_4$ (757 mg, 0.65 mmol, 5 mol%), the atmosphere was exchanged for dry N_2. Next, anhydrous DMF (26 mL), diisopropylamine (3.7 mL, 26 mmol, 2 eq.), 1-bromo-1-propene (**4**, 60:40 mixture of *E*/*Z* isomers, 1.4 mL, 16 mmol, 1.2 eq.) and pent-4-yn-1-ol (**5**, 1.3 mL, 13 mmol, 1 eq.) were added to the flask in this order, at room temperature. The reaction was stirred for 24 h at room temperature, then H_2O (200 mL) and Et_2O (200 mL) were added, the mixture was shaken vigorously, the organic phase was separated, washed with H_2O (100 mL) and brine (100 mL), dried ($MgSO_4$) and concentrated *in vacuo* (35 °C, 600-50 mbar). A sample was taken to measure the *E*/*Z* ratio of the product (ranging from 93:7 to >95:5 of *E*/*Z* ratio). Next, the crude was purified by flash column chromatography (SiO_2, hexanes:EtOAc, 85:15 to 75:25) to furnish alcohol **2** (862 mg, 6.9 mmol) as an orange-brown oil in 53% yield. R_f 0.42 (SiO_2, hexanes/EtOAc 60:40). ^{1}H NMR (250 MHz, $CDCl_3$): δ 1.67-1.85 (m, 5H), 1.96 (s, 1H), 2.38 (td, *J* = 6.9, 1.7 Hz, 2H), 3.72 (t, *J* = 6.2 Hz, 2H), 5.43 (dsext, *J* = 15.8, 1.9 Hz, 1H), 6.04 (dq, *J* = 15.8, 6.8 Hz, 1H). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 15.8 (CH_2), 18.3 (CH_3), 31.3 (CH_2), 61.7 (CH_2), 79.7 (C), 87.4 (C), 110.8 (CH), 138.3 (CH). Data obtained for the (*Z*)-isomer: ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.57 (s, 1H), 1.81 (quint, *J* = 6.5 Hz, 2H), 1.85 (dd, *J* = 6.8, 1.7 Hz, 3H), 2.49 (td, *J* = 6.9, 2.1 Hz, 2H), 3.79 (t, *J* = 6.1 Hz, 2H), 5.45 (d sext, *J* = 10.7, 1.9 Hz, 1H), 5.90 (dq, *J* = 9.7, 6.8 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 15.7 (CH_3), 16.1 (CH_2), 31.5 (CH_2), 61.9 (CH_2), 77.8 (C), 93.8 (C), 110.1 (CH), 137.3 (CH).

*4.1.3. (4Z,6E)-octa-4,6-dien-1-ol (**9**)*

Zinc dust (5.00 g, 76 mmol, 76 eq.) and H_2O (30 mL) were added to a round-bottomed flask, and argon was bubbled through the mixture with stirring at room temperature for 15 min. Next, the bubbling was stopped and the argon atmosphere was maintained, $Cu(OAc)_2$•H_2O (1.00 g, 4.9 mmol, 4.9 eq.) was added by removing the septum and adding the solid quickly and the reaction mixture allowed to stir for another 15 min. Then $AgNO_3$ (600 mg, 3.5 mmol, 3.5 eq.) was added by removing the septum and adding the solid quickly. This step is exothermic and temperature of the reaction mixture was slightly increased. The reaction was stirred for 30 min. Then the solid was separated by filtration under a stream of nitrogen, and was washed with H_2O (3 x 25 mL), methanol (3 x 25 mL), acetone (3 x 25 mL), and Et_2O (3 x 25 mL). The alloy still moist with Et_2O was collected and added to a solution of alkyne **2** (124 mg, 1.0 mmol, 1 eq.) diluted in MeOH/H_2O (15 mL, 1:1) under a N_2 atmosphere, and TMSCl (1.3 mL, 10 mmol, 10 eq.) was added. The reaction was stirred for 30 h and a second portion of TMSCl (1.3 mL, 10 mmol, 10 eq.) was added. The reaction was stirred for a second period of 30 h, then the reaction mixture was filtered through a column of Celite and the solid was washed with EtOAc (4 x 80 mL). The liquid phase was washed with brine (50 mL), the organic phase was dried ($MgSO_4$), concentrated *in vacuo* and the crude product was purified by flash chromatography (SiO_2, hexanes:EtOAc, 75:25) to furnish the diene **9** (74.4 mg, 0.59 mmol) as a colorless oil in 59% yield. R_f 0.46 (SiO_2, hexanes/EtOAc 60:40). ^{1}H NMR (250 MHz, $CDCl_3$): δ 1.51 (br. s, 1H), 1.66 (quint., *J* = 7.0 Hz, 2H), 1.77 (d, *J* = 6.5 Hz, 3H), 2.25 (q, *J* = 7.2 Hz, 2H), 3.66 (t, *J* = 6.5 Hz, 2H), 5.29 (dt, *J* = 10.7, 7.6 Hz, 1H), 5.68 (dq, *J* = 14.9, 6.6 Hz, 1H), 5.97 (t, *J* = 10.9 Hz, 1H), 6.04 (ddt, *J* = 15.0, 11.1, 1.3 Hz). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 18.7 (CH_3), 23.9 (CH_2), 32.4 (CH_2), 62.2 (CH_2), 126.7 (CH), 128.5 (CH), 129.1 (CH), 129.5 (CH).

*4.1.4. Spilanthol (**1**)*

A round-bottomed flask under a N_2 atmosphere was charged with dry CH_2Cl_2 (8 mL) and oxalyl chloride (144 µL, 1.7 mmol, 2 eq.). The solution was cooled to −78 °C, and anhydrous DMSO (181 µL, 2.5 mmol, 3 eq.) was added (evolution of gas was observed). The reaction was stirred for 15 min, then alcohol **9** (107 mg, 0.85 mmol, 1 eq.) was added to the mixture as a solution in CH_2Cl_2 (2 mL + 2 mL of washing). The resulting solution was stirred for 1 h at −78 °C. Next, dry Et_3N (711 µL, 4.2 mmol, 6 eq.) was added, the cooling bath was removed and the reaction was further stirred for 30 min. The reaction was diluted with Et_2O (15 mL), and the organic phase was extracted with H_2O (2 x 10 mL), brine (10 mL), dried ($MgSO_4$), and concentrated under reduced pressure (100 mbar, 25 °C, due the volatility of aldehyde). The crude product was used in the HWE step without further purification.

Phosphonate **3** (278 mg, 1.1 mmol, 1.3 eq.) was placed in a round-bottom flask with a stirrer bar, the atmosphere was exchanged for dry N_2 and anhydrous THF (10 mL) was added. Next, the solution was cooled to 0 °C, and NaH in mineral oil

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(60% w/w, 170 mg, 4.2 mmol, 5 eq.) was added by removing the septum and adding the solid quickly. Then, crude aldehyde (from Swern step) was added to the reaction as a solution in THF (2 mL + 2 mL of washing), the cooling bath was removed, and the reaction was stirred for 30 min. The reaction was quenched by addition of aqueous HCl solution (1 M, 10 mL), the mixture was extracted with EtOAc (15 mL), and the organic phase was washed with brine (15 mL), dried ($MgSO_4$) and concentrated under reduced pressure. The product was purified by flash chromatography (SiO_2, hexanes:EtOAc, 75:25) to furnish spilanthol (**1**, 118 mg, 0.53 mmol) as a colorless oil in 63% yield. R_f 0.20 (SiO_2, hexanes/EtOAc 75:25). FTIR (ATR, cm^{-1}): 3280, 2957, 2927, 2870, 1668, 1627, 1545, 1235, 1159, 978, 944, 818, 618. ^{1}H NMR (600 MHz, $CDCl_3$): δ 0.89 (d, *J* = 6.8 Hz, 6H), 1.70 (d, *J* = 6.4 Hz, 3H), 1.70-1.81 (m, 1H), 2.23 (q, *J* = 7.2 Hz, 2H), 2.29 (q, *J* = 7.2 Hz, 2H), 3.12 (t, *J* = 6.3 Hz, 2H), 5.23 (dt, *J* = 10.3, 7.5 Hz, 1H), 5.67 (dq, *J* = 14.7, 7.0 Hz, 1H), 5.81 (d, *J* = 15.2, 1H), 5.78-5.88 (m, 1H), 5.94 (t, *J* = 10.9, 6.8 Hz, 1H), 6.26 (dd, *J* = 13.4, 12.6 Hz, 1H), 6.80 (dt, *J* = 15.3, 7.0 Hz, 1H). ^{13}C NMR (151 MHz, $CDCl_3$): δ 18.3 (CH_3), 20.1 ($2CH_3$), 26.4 (CH_2), 28.6 (CH), 32.1 (CH_2), 46.9 (CH_2), 124.2 (CH), 126.7 (CH), 127.6 (CH), 129.4 (CH), 129.9 (CH), 143.5 (CH), 166.1 (C). HRMS (ESI+): *m/z* calculated for $C_{14}H_{24}NO^+$ $[M+H^+]$ 222.1852, found 222.1846.

*4.1.5. (2E,8E)-N-isobutyldeca-2,8-dien-6-ynamide (**8**)*

A round-bottomed flask under a N_2 atmosphere was charged with dry CH_2Cl_2 (10 mL) and oxalyl chloride (169 µL, 2.0 mmol, 2 eq.). The solution was cooled to −78 °C, and anhydrous DMSO (213 µL, 3.0 mmol, 3 eq.) was added (evolution of gas was observed). The reaction was stirred for 15 min, then alcohol **2** (124 mg, 1.0 mmol, 1 eq.) was added to the reaction as a solution in CH_2Cl_2 (2 mL + 2 mL of washing). The resulting solution was stirred for 1 h at −78 °C. Next, dry Et_3N (836 µL, 6.0 mmol, 6 eq.) was added, the cooling bath was removed, and the reaction was further stirred for 30 min. The reaction was diluted with Et_2O (20 mL), and the organic phase was extracted with H_2O (2 x 10 mL), brine (10 mL), dried ($MgSO_4$), and concentrated *in vacuo* (120 mbar, 25 °C, due the volatility of aldehyde). The product was used in the HWE step without further purification.

Phosphonate **3** (327 mg, 1.3 mmol, 1.3 eq.) was placed in a round-bottomed flask with a stirrer bar, the atmosphere was exchanged for dry N_2 and anhydrous THF (10 mL) was added. Next, the solution was cooled to 0 °C, and NaH in mineral oil (60% w/w, 200 mg, 5 mmol, 5 eq.) was added by removing the septum and adding the solid quickly. Then crude aldehyde obtained was added to the reaction as a solution in THF (2 mL + 2 mL of washing), the cooling bath was removed, and the reaction was stirred for 30 min. The reaction was quenched by addition of NH_4Cl saturated solution (15 mL), the mixture was extracted with EtOAc (15 mL), and the organic phase was washed with brine (15 mL), dried ($MgSO_4$) and concentrated under reduced pressure. The crude product was purified by flash column chromatography (SiO_2, hexanes:EtOAc, 75:25 to 60:40) to furnish the amide **8** (112 mg, 0.51 mmol) as a white solid in 51% yield. R_f 0.15 (SiO_2, hexanes/EtOAc 75:25). FTIR (ATR, cm^{-1}): 3291, 2959, 2914, 2870, 1666, 1625, 1545, 1334, 1236, 1220, 950, 650. ^{1}H NMR (250 MHz, $CDCl_3$): δ 0.87 (d, *J* = 6.6 Hz, 6H), 1.70 (dd, *J* = 6.3, 1.6 Hz, 3H), 1.70-1.84 (m, 1H), 2.30-2.45 (m, 4H), 3.09 (t, *J* = 6.3 Hz, 2H), 5.43 (dq, *J* = 15.8, 1.7 Hz, 1H), 5.85 (d, *J* = 15.3, 1.7 Hz, 1H), 6.00 (dq, *J* = 15.6, 6.8 Hz, 1H), 5.96-6.12 (m, 1H), 6.69-6.84 (dt, *J* = 15.3, 6.4 Hz, 1H). ^{13}C NMR (62.9 MHz, $CDCl_3$): δ 18.3 (CH_3), 18.4 (CH_2), 20.0 ($2CH_3$), 28.4 (CH), 31.2 (CH_2), 46.8 (CH_2), 79.9 (C), 86.6 (C), 110.7 (CH), 124.7 (CH), 138.4 (CH), 141.8 (CH), 165.8 (C). HRMS (ESI+): *m/z* calculated for $C_{14}H_{22}NO^+$ $[M+H^+]$ 220.1696, found 220.1710.

4.2. Biological assays

All reagents and solvents were analytical grade. A eutectic mixture of 2.5% lidocaine and 2.5% prilocaine (5% EMLA® cream) was obtained from AstraZeneca (São Paulo, Brazil).

4.2.1. Bioadhesive film production

Hydroxyethyl cellulose (2.5 g) was added to distilled water (100 mL) under heating (55 ± 2 °C) and stirring, until a translucent gel was obtained. Agitation was stopped until the mixture reached room temperature. Next, Tween® 80 (0.7 g), glycerin (0.7 g) and Transcutol® (2.5 g) were added and homogenized. Spilanthol (**1**, 4.14 mg, 18.7 µmol) or analogue **8** (4.12 mg, 18.8 µmol) were diluted with methanol (maximum of 0.5 mL) and the solutions were added to a portion of 30 g of gel. The resulting mixtures were put on Nylon molds, and these films were dried for 24 h at 40 °C. Then, the films were cut with a circular punch (17 mm of diameter).

4.2.2. In vitro permeation studies

In vitro permeation studies were carried out using jacketed Franz-type vertical diffusion cells (Manual Diffusion Test System l, Hanson Research Corporation, Chatsworth, CA, USA) with a permeation area of 1.77 cm^2 and a receptor compartment volume of 7 mL. The jacket was coupled to a water bath (Fisher Scientific®) at 32 °C. Dermatomed pig ear skin (Nouvag AG), obtained from a local slaughterhouse (Frigorífico Angelelli, located in Piracicaba, SP, Brazil, 22°40'09.2"S 47°40'26.6"W) was used as the barrier in all experiments. The skin was excised from the outer part of the ear, separated from the underlying cartilage using a scalpel, dermatomed up to 500 µm thick, and frozen at −20 °C until used.[55,60] Bioadhesive and skin were clamped between the donor and receptor compartments. The bioadhesives were applied in finite dose conditions,, saline-methanol (70:30, v/v) solution was used in the receptor compartment to ensure Sink conditions and maintained under constant magnetic stirring (350 rpm). Samples (400 µL) were periodically withdrawn from the receptor compartment and immediately replaced by the same volume of solution, taking account of dilution effects.

The samples were transferred to chromatography vials for quantification of spilanthol and analogue by HPLC analysis using a Waters Alliance HPLC-DAD system (Waters Corp., Milford, MA, USA) operating with an XTerra® MS C18 column (150 mm x 2.1 mm i.d., 5 µm; Waters) at 40 °C. The mobile phase consisted of acetonitrile/water 40:60 v/v, with a flow rate of 0.3 mL/min. Absorbance was monitored at 232 nm. The quantification of spilanthol and analogue **8** was conducted using external calibration curve with 6 points at a concentration range of 2 to 50 $\mu g.mL^{-1}$, using methanol as diluent. The volume of injection was 20 µL. All solutions were filtered through a 0.45 µm PVDF membrane before analysis.

The cumulative amount of spilanthol or analogue **8** transported across the skin was plotted as a function of time. Their flux across the barrier was calculated from the slope of the linear portion of the curve and the lag time was obtained from the intercept with the time axis.

4.2.3. Anesthetic effect evaluation in vivo

Swiss male mice (25-40 g) from Multidisciplinary Center of Biological Investigation of Laboratory Animals (CEMIB-University of Campinas) were maintained at 22 ± 2 °C under light/dark cycles of 12 h. Approval for these assays was provided by the Animal Ethics Committee of University of Campinas

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(protocol #4137-1). The mice were divided into groups of 6 animals.

Topical anesthetic efficacy of bioadhesive films containing spilanthol (**1**) or analogue **8** was evaluated with the tail flick assay. The mouse was placed in an acrylic restraint while the distal portion of its tail (10 cm) was maintained free (see Supporting Information). The tail was submitted to irradiation from an incandescent lamp (55 °C), and the time necessary for tail removal (latency) was considered as an aversive response to heat. The baseline was recorded for each animal before the beginning of the experiment, and only those with baselines below 6 s were considered. In order to avoid tissue damage, the maximal time for irradiation of the tail was established as 10 s (cut-off value). Bioadhesive films containing spilanthol (**1**) or analogue **8** were compared to EMLA® (150 mg/animal, 7.5 mg of anesthetic), as a positive control, and the bioadhesive film without anesthetic, as a negative control. The films or EMLA® were applied at 2 cm from the tail base for 5 min. The tested substances were removed, and the nociceptive stimulus was applied to the same region immediately, and then every 15 min until the animal returned to its baseline pain response. The duration of analgesia was defined as the increase in required time for withdrawal of the tail, which was at least 50% higher than the baseline value. The data were reported as the percentage of the maximum possible effect (MPE, in minutes), which was calculated from the following relation.

%MPE = [(test latency − baseline latency)/(cut-off time − baseline latency) x 100], area under the curve was recorded for each experimental group.

Competing interests

The authors declare the following competing financial interests: two patents have been filled on the synthetic route of spilanthol and analogues (BR1020160178711) and on an anesthetic formulation and its use (BR10201701137).

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Supplementary Material

^{1}H, DEPT135 and ^{13}C NMR spectra for all synthesized compounds. Details for bioadhesive films preparation, *in vitro* permeation studies and tai flick assay. Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.tetxxxxxxxxx.

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