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Structure–activity relationships of the ultrapotent vanilloid resiniferatoxin (RTX): The homovanillyl moiety

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Abstract—Starting from ROPA (**2**), analogues of RTX (**1a**) modified on the acyl side chain were prepared and evaluated for vanilloid activity in HEK-293 cells over-expressing the human recombinant TRPV1. The ROPA motif provided an enhancement of potency sufficient to expand the range of vanillyl surrogates to structural elements (e.g., an unsubstituted phenyl ring) that afford inactive analogues in compounds from the capsaicin series.
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The daphnane diterpenoid resiniferatoxin (RTX, **1a**) was discovered in 1975 because of its extraordinary activity in the mouse ear reddening assay, a surrogate end-point for mouse skin tumor promotion.[1] In this assay, RTX outperforms PMA (phorbol myristate acetate), the most potent phorbol ester, by 2–3 orders of magnitude.[1] However, since RTX lacks tumor promoting properties, its biological target remained elusive.[2] An analogy with capsaicin was suggested by the revision of the substitution pattern of the side-chain aromatic moiety of RTX. This aryl group was originally formulated as a 3-methoxy-5-hydroxyphenyl substituent, but was later recognized as a vanillyl (3-methoxy-4-hydroxyphenyl) group,[3] and in 1989 it was indeed demonstrated that RTX behaves as an ultrapotent analogue of capsaicin, the pungent principle of hot peppers.[4] This seminal discovery established RTX as an indispensable neuropharmacological tool, paving the way for the characterization and cloning of the long sought capsaicin (vanilloid) receptor (TRPV1),[5] and eventually establishing the burgeoning field of TRP channel research.[6] RTX has also been investigated as a drug for the treatment of a series of conditions (lower urinary tract disfunctions, neuropathic and cancer pain) where malfunctioning or over-expression of TRV1 is involved.[7] Very promising results were recently reported in this direction,[8] while RTX was further validated as a privileged and unique structure to investigate the biology of TRPV1 by the serendipitous discovery that its powerful agonistic activity on TRPV1 (EC_{50} ca. 19 pM) could be reversed by aromatic iodination of the acyl moiety, affording the ultrapotent vanilloid antagonist iodoresiniferatoxin (I-RTX, **1b**).[9]

Research on RTX has been hampered by the limited availability of the natural product. As a result, our knowledge on its structure–activity relationships is still fragmentary,[10] and nothing is known on the possibility to re-direct RTX, the most potent chemical activator of a TRP channel reported to date, to other members of this large family of sensors, for many of which no chemical activator is known.[6]

Keywords: Resiniferatoxin (RTX); TRPV1; Structure–activity relationships; Capsaicin; Vanilloids.
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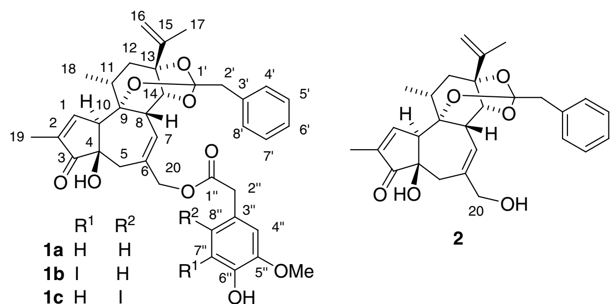
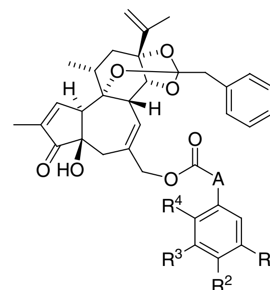


We have recently developed an expeditious protocol to obtain the terpenoid core of RTX (resiniferonol orthophenylacetate, ROPA, **2**), from *Euphorbia resinifera* Berg, a household plant commercially available from nurseries.[11] Capitalizing on the availability of 'synthetic' amounts of ROPA, we have investigated the structure–activity relationships of the critical homovanillyl moiety of RTX, focusing on the aliphatic linker between the ester carbonyl and the aryl moiety, and on the substitution pattern of the phenyl ring. While not exhaustive, these modifications were expected to shed light on a series of critical issues, like the optimal distance between the vanillyl moiety and the diterpenoid core, the role of the aryl oxygen functions, and the generality of the switch in vanilloid activity observed upon aryl iodination.[12]

All RTX analogues were prepared by Mitsunobu esterification (DIAD–TPP as the redox couple) of ROPA with the corresponding carboxylic acids.[13] Purification of the reaction products was greatly facilitated by the crystallization of a diisopropyl hydrazodicarboxylate–triphenylphosphine oxide adduct by cooling in toluene.[14] After filtration and gravity column chromatography, the final products were further purified by crystallization or, if amorphous, by preparative HPLC on silica gel.[15] Final compounds were prepared in 10–20 mg scale (30–60% overall yield) and showed HPLC purity >96%. We found this protocol more convenient, higher yielding, and of more general applicability than the other methods described in the literature for the esterification of ROPA (Steglich esterification,[10] Yamaguchi acylation,[16] Mukayama coupling,[3,17] and reaction of 20-mesyl ROPA with sodium carboxylates[18]).

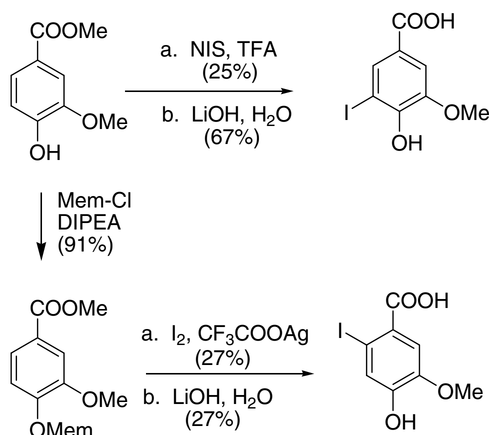


	A	R¹	R²	R³	R⁴
3a	-	OMe	OH	H	H
3b	$-CH_2-CH_2-$	OMe	OH	H	H
3c	(*E*) –CH=CH-	OMe	OH	H	H
3d	-	H	H	H	H
3e	$-CH_2$	H	H	H	H
3f	$-CH_2-CH_2-$	H	H	H	H
3g	(*E*) –CH=CH-	H	H	H	H
3h	-	OMe	OH	I	H
3i	-	OMe	OH	H	I
3j	$-CH_2-CH_2-$	OMe	OH	I	H
3k	$-CH_2-CH_2-$	OMe	OH	H	I

The iodinated vanillic and dihydroferulic acids required for this study were prepared from the corresponding acids as exemplified in Scheme 1 for 5- and 6-iodovanillic acids. In RTX, the effect of iodination depends on the location of the halogen atom on the homovanillyl moiety, with the 5′-iodo analogue (**1b**) being an ultrapotent antagonist[9] and the 6′-derivative (**1c**) a mixed agonist.[19] The purity of the starting iodovanillic acids was therefore of utmost relevance, and the low yields of both iodination steps reflect the extensive purification by chromatography and/or crystallization of the reaction products required to secure the absence of detectable (300 MHz ^{1}H NMR spectroscopy) amounts of starting material or isomers in the final product. The regiochemistry of the iodination step was controlled by the nature of the oxygen function at C-4. While a free hydroxyl at C-4 directed iodine to the adjacent carbon, etherification of the phenolic hydroxyl led to the complementary C-6 functionalization, as observed for vanillin.[12]

Final products (**3a**–**3k**) were assayed in human embryonic kidney (HEK)-293 cells over-expressing the human TRPV1, and the results are reported in Table 1.[20]

Structure–activity data of RTX are not only scarce, but also difficult to compare, not only because of differences in the type of vanilloid assay employed (functional or binding), but also because different versions of TRPV1 show different sensitivity to vanilloids, with hTRPV1 being less sensitive to RTX compared to its murine version (rTPRV1).[21] Furthermore, differences have also been noticed between assays carried out in native dorsal root ganglion cells and in cells transfected with



Scheme 1. Synthesis of 5- and 6-iodovanillic acids (NIS = *N*-iodosuccinimide; TFA = trifluoroacetic acid; Mem = methoxyethoxymethyl; DIPEA = diisopropylethylamine).

Table 1. Vanilloid agonistic activity of aryl substituted vanillamides[20]

Compound	EC_{50}[a] (nM)
1a (RTX)	0.019
3a	9.4
3b	4.6
3c	1.5
3d	25.5
3e	4.7
Capsaicin	40.0
3f	51.5
3g	69.5
3h	1.9
3i	15.2
3j	8.6
3k	7.9

[a] EC_{50}, concentration exerting a half-maximal effect. Data are means of $n = 3$ determinations, whose SD values were never higher than 10% of the means.

cloned TRPV1.[21] It was therefore important to adopt a homogeneous end-point, and this study represents the first attempts to explore in a systematic way the structure–activity relationships of RTX using hTRPV1. Comparison of the activity of RTX with its vanillyl-, coniferyl-, and dihydroconiferyl analogues showed that the nature of the linker between the vanillyl and the ester carbonyl is critical for ultrapotency. Thus, deletion, homologation, or replacement with a double bond were all detrimental for activity, with a decrease of potency in the range of 2–2.5 orders of magnitude. Lengthening of the linker increased activity compared to its deletion by 3-fold (homologation) and 6-fold (replacement with a double bond). Nevertheless, given the ultrapotency of RTX, all these compounds (**3a**–**3c**) would qualify as potent vanilloids. Somewhat similar results were observed for the removal of the oxygen functions of the vanillyl moiety of RTX. This maneuver was detrimental for activity, with a ca. 200-fold decrease of potency. Nevertheless, with an EC_{50} in the one digit nanomolar range (4.7 nM), ROPA phenylacetate (**3d**) still showed potent vanilloid activity, one order of magnitude higher than capsaicin. Remarkably, removal of the oxygen functions in the analogues from the coniferyl- and dihydroconiferyl series was better tolerated, while, compared to ROPA vanillate (**3a**), ROPA benzoate (**3e**) even showed a 2-fold increase of activity. Taken together, these observations suggest that the ROPA motif provides an enhancement of potency sufficient to expand the range of vanillyl surrogates to structural elements, like an unsubstituted phenyl ring, that instead afford inactive analogues in compounds from the capsaicin series.[22] For the same reason, maneuvers like homologation, that are incompatible with the vanillyl moiety of capsaicin,[22] are possible with resiniferonoids. The ultrapotency of RTX clearly requires the well-defined topologic and constitutional relationship between the side-chain aromatic moiety and the diterpenoid core expressed by the homovanillyl group. Nevertheless, a certain flexibility must exist in the binding pocket of TRPV1, making it possible to accommodate a range of acyl chains that, in the absence of ROPA, would be incompatible with binding.

Iodination of compounds from the vanillic and the dihydroferulic series gave surprising results, since, in sharp contrast with what was observed in the homovanillic analogue (RTX), no reversal of activity was observed, and all compounds behaved as full agonists. Interestingly, iodination of vanillyl ROPA (**3a**) in *ortho* to the phenolic hydroxyl (**3h**) caused a 5-fold increase of activity, while introduction of an iodine atom at the C-6 *meta*-position (**3i**) caused an almost 2-fold decrease of activity. Both iodinated analogues of dihydroferuloyl ROPA (**3k** and **3l**) were 2-fold less potent than their parent compound (**3b**).

Information on the vanilloid binding pocket of TRPV1 has been mostly obtained by experiments of oligonucleotide-directed mutagenesis.[23] The availability of ROPA makes it possible to purse a complementary and more traditional approach, based on the structure–activity relationships of the ultrapotent ligand RTX. In this first contribution, we have shown that, while the methylene linker and the oxygen functions on the side chain of RTX are essential for ultrapotency, the presence of the diterpenoid core makes it possible to perform a surprising range of side-chain modifications, including removal of the metabolically labile guaiacyl oxygens. Furthermore, the lack of iodine-induced reversal of activity observed in the lower- and higher-homologues of RTX points to a topologically well-defined role for this atom in the ultrapotent vanilloid antagonist I-RTX (**1b**).

Acknowledgment

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