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The Effect of Counterion and Tertiary Amine on the Efficiency of *N*-Triazinylammonium Sulfonates in Solution and Solid-Phase Peptide Synthesis

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A collection of *N*-triazinylammonium sulfonates, designed according to the concept of "superactive esters", was obtained by treatment of ammonium sulfonates with 2-chloro-4,6-dimethoxy-1,3,5-triazine. The structure of the tertiary amine as well as sulfonate anion influenced their reactivity and stability in *N*,*N*-dimethylformamide (DMF) solution. The reagents were successfully used in solution- and solid-phase synthesis of Z-, Boc-, and Fmoc-protected peptides containing natural and unnatural sterically hindered amino acids as well as in [2+1] fragment condensation approaches, yielding the final products in 80–100 % yield and high optical purity. In manual SPPS of the $[Aib]^2[Aib]^4$-enkephalin analogue and the ACP(65–74) peptide fragment VQAAIDYINEG, several sulfonates yielded peptides significantly faster than TBTU or HATU. Comparative analyses demonstrated that 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium 4-toluenesulfonate was the most versatile reagent in a wide range of coupling procedures.

Introduction

The increasing pool of naturally occurring peptides bearing noncoded amino acid residues, peptides decorated with additional structural elements, as well as benefits attended by incorporation of unnatural building blocks into native peptide chains, provide strong motivation for the systematic search for new coupling reagents and more efficient procedures.[1] To overcome problems caused by the instability and poor reactivity of some building blocks and to improve the process of peptide synthesis, we developed an innovative coupling strategy, avoiding over-activation. This novel strategy was achieved by introducing an additional, synchronous, and thermodynamically highly favoured process into the synthetic pathway leading to peptide (i.e., amide) bond formation. Thus, this approach prevents (or at least reduces) side reactions and racemisation at the stereogenic centre brought about by strong activation of the carboxylic function. The previously[2] developed triazine-based coupling reagents (TBCRs) display the structural features we postulated for this novel concept.[3] Previous studies proved the efficacy of 2-acyloxy-1,3,5-triazines **1** ("superactive esters")[3] as acylating intermediates and confirmed the enhanced propensity of the triazinyl moiety as a leaving group in the process of amine acylation.[4] This characteristic was attributed to the energetically favoured isomerisation of 2-hydroxy-1,3,5-triazine **3** to the appropriate triazinone **4** (Figure 1).

In this way, the triazinyl "superactive ester" **1** activation "push" was amplified by the "pull" caused by additional stabilisation of the side-product **3** by its transformation into thermodynamically more stable **4**. This was expected to give better synthetic results than those obtained solely due to more powerful activation. The TBCRs that were designed according to this concept were demonstrated to be useful in the synthesis of amides,[5,6,7] esters,[8] and anhydrides of carboxylic acids,[9] in peptide and glycopeptide synthesis by conventional and microwave-assisted strategies,[6,10] in the synthesis of seleno- and thiono-phosphoranes analogues of nucleic acid,[11] and in many other synthetic procedures. The modular structure of TBCRs allows extensive modification of their structure to fine-tuning their properties in user-friendly forms, at a reasonably low cost and low hazard levels. There are three ways to modulate the properties of triazine-based condensing reagents encompassing: incorporation of substituents on the triazine ring, structurally diversification of the ammonium fragments, and modification of the counterion structures.

Thus, introduction of substituents on the triazine ring such as highly fluorinated hydrocarbon side chains led to fluorinated coupling reagents designed for peptide synthesis in fluorophorous media.[12] Moreover, hydrophilic triazines were successfully used for peptide bond formation in aqueous solution,[13] whereas the introduction of a benzyl group

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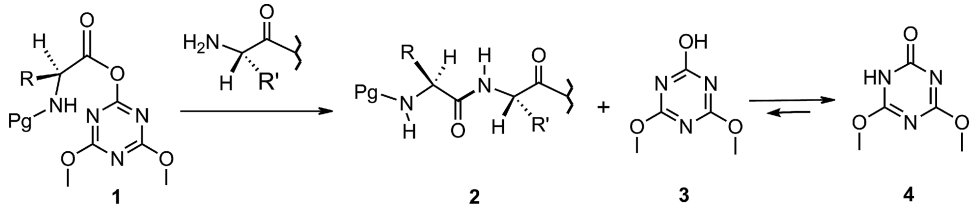


Figure 1. Peptide bond formation by using triazine-based coupling reagents is accompanied by energetically favoured isomerisation of 2-hydroxy-1,3,5-triazine **3** into the more stable triazinone **4**.

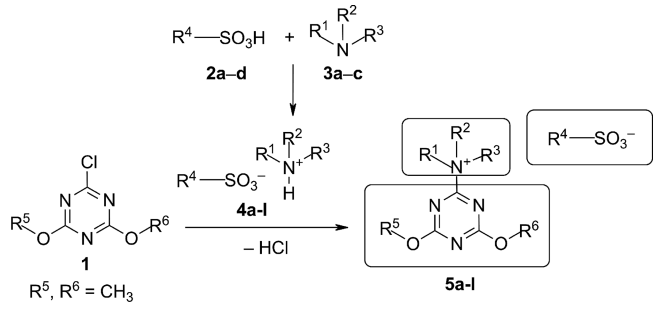
into the triazine ring enhanced reactivity in the case of SPPS on polystyrene resins.[14] The advantageous properties of triazines have been confirmed by designing and synthesising *N*-methyl-*N*-triazinylmorpholinium tetrafluoroborate, which outperformed *N*-hydroxybenzotriazole-based reagents in terms of reactivity and stability in a broad range of possible applications.[15] Further structural improvements based on replacing methoxyl groups of the triazine ring with 2,2,2-trifluoroethoxyl substituents substantially enhanced the efficiency of new *N*-triazinylmorpholinium tetrafluoroborate analogues in the synthesis of extremely difficult sequences, such as MeVal-MeVal and MeLeu-MeLeu, in the enkephalin analogues TyrMeValMeValPheLeu and TyrMeLeuMeLeuPheLeu, making them much more efficient than the classic benzotriazole-based coupling reagents[16] and comparable only with Oxyma-based reagents.[17]

With these considerations in mind, we focused our attention on increasing the versatility of *N*-triazinylammonium reagents by replacing the small, nonpolarisable tetrafluoroborate anion or hexafluorophosphate with the more lipophilic alkyl(aryl)sulfonate counterion. Most sulfonate anions are stable, inexpensive, environmentally friendly, and readily available in a broad range of structural forms. In our preliminary report,[18] it was confirmed that a wide collection of *N*-triazinylammonium sulfonates were easily affordable. Herein, we present a systematic study in order to establish how modifications of the tertiary amine component and counterion could influence the stability, versatility and coupling efficiency of this family of coupling reagents. This proposition was additionally supported by the previously reported influence of the counterion on the stability of coupling reagents[19a] as well as disruption of the peptide chain conformation in the presence of the ions.[19b]

Results and Discussion

The reagents **5a**–**l** were obtained by treatment of 2-chloro-4,6-dimethoxy-1,3,5-triazine (**1**) with sulfonates of the tertiary amines **4a**–**l** in the presence of sodium hydrogen carbonate.[20]

Taking advantage of the modular structure of triazine reagents, **5a**–**l** were prepared starting from the weakly basic *N*-methylmorpholine (**3a**), as well as the more basic *N*-methylpyrrolidine (**3b**) and *N*-methylpiperidine (**3c**). As counterions, we selected the moderately lipophilic and highly polarisable 4-toluenesulfonic acid (**2a**), the lipophilic but nonpolarisable 10-camphorsulfonic acid (**2b**), the hydrophilic nonpolarisable methanesulfonic acid (**2c**), and the extremely acidic, non-nucleophilic trifluoromethanesulfonic acid (**2d**) (see Scheme 1, Figure 2).



Scheme 1. Synthesis of *N*-(4,6-dimethoxy-1,3,5-triazin-2-yl)-*N*,*N*,*N*-trisubstituted ammonium sulfonates **5a**–**l**.

The new TBCRs **5a**–**l** were obtained in 55–99% yield as white solids isolated after crystallisation in acetonitrile/diethyl ether or as oils, and used in coupling reactions without additional purification (see Table 1).

As expected, in all cases the activation of 4-methoxybenzoic acid (**6**) with **5a**–**l** gave the triazine "superactive ester" **7**, which was isolated in 64–98.5% yield (see Scheme 2 and Table 2). Its structure was confirmed by comparison with an authentic sample obtained by using the classical procedure and by the presence of the characteristic IR band at 1750–1780 cm^{-1}.

To determine the compatibility of the new TBCRs **5a**–**l** in solution and solid-phase peptide synthesis, both by manual and automatic strategies, we evaluated their solubility and stability by HPLC and ^1H NMR techniques.

As expected, it was found that all the sulfonates **5a**–**l**, both solid and oil compounds, were stable enough to be stored at low temperature for at least six months, and, moreover, in the case of DMT/NMM/TsO$^-$ (**5a**) NMR studies after one year of storage at 4 °C confirmed their stability. However, their stability in *N*,*N*-dimethylformamide (DMF) solution varied at room temperature (see Tables 3 and 4).

The most stable compounds were the most lipophilic *N*-triazinylammonium 4-toluenesulfonates **5a**,**e**,**i** and 10-camphorosulfonates **5b**,**f**,**j**, and their stability was comparable to that of conventional coupling agents such as TBTU, HBTU and COMU. On the other hand, *N*-triazinylammonium methanesulfonates **5c**,**g**,**k** were less stable, which restricts

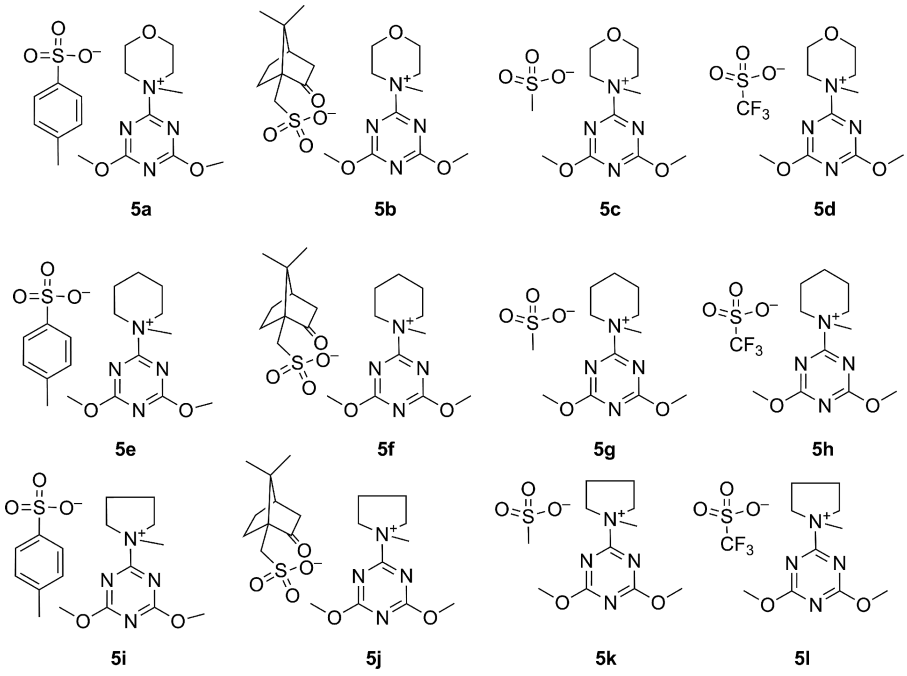
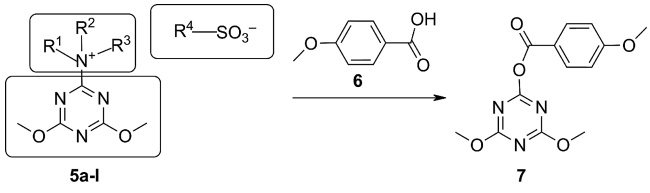


Figure 2. The new collection of TBCRs, sulfonates of the *N*-triazinylammonium salts **5a**–**l**.

Table 1. Characteristics of sulfonates **5a**–**l**.

Entry	Sulfonates of *N*-triazinylammonium salts **5**		Yield [%]	M.p. [°C]
1	DMT/NMM/TsO$^-$	**5a**	72	57–61
2	DMT/NMM/CsO$^-$	**5b**	96	127–129
3	DMT/NMM/MsO$^-$	**5c**	55	96–98
4	DMT/NMM/TfO$^-$	**5d**	86	112–114
5	DMT/NMPip/TsO$^-$	**5e**	99	oil
6	DMT/NMPip/CsO$^-$	**5f**	86	110–115
7	DMT/NMPip/MsO$^-$	**5g**	87	oil
8	DMT/NMPip/TfO$^-$	**5h**	98	85–90
9	DMT/NMPyr/TsO$^-$	**5i**	99	81–85
10	DMT/NMPyr/CsO$^-$	**5j**	84	oil
11	DMT/NMPyr/MsO$^-$	**5k**	84	oil
12	DMT/NMPyr/TfO$^-$	**5l**	94	85–90



Scheme 2. Synthesis of triazine "superactive ester" **7** from 4-methoxybenzoic acid (**6**) and TBCRs **5a**–**l**.

their possible use in automatic SPPS. As expected, stability of **5a**–**l** in DMF solution further decreased in the presence of a stoichiometric amount of DIPEA. In solution, in the presence of a base (Table 4), the most stable compounds were again *N*-triazinylammonium 4-toluenesulfonates **5a**,**e**,**i** and trifluoromethanesulfonates **5d**,**h**,**l** but not 10-camphorsulfonates **5b**,**f**,**j** or *N*-triazinylammonium methanesulfonates **5c**,**g**,**k**. In the case of the classical coupling reagents

Table 2. Activation time and yield in the synthesis of 4,6-dimethoxy-1,3,5-triazin-2-yl 4-methoxybenzoate (**7**) using the new TBCRs **5a**–**l** and DMT/NMM/BF$_4^-$.[15]

Entry	Coupling reagent		Activation time [min]	Yield [%]
1	DMT/NMM/TsO$^-$	**5a**	30	98.5
2	DMT/NMM/CsO$^-$	**5b**	30	76.1
3	DMT/NMM/MsO$^-$	**5c**	30	97.0
4	DMT/NMM/TfO$^-$	**5d**	30	90.0
5	DMT/NMPip/TsO$^-$	**5e**	30	98.0
6	DMT/NMPip/CsO$^-$	**5f**	30	79.2
7	DMT/NMPip/MsO$^-$	**5g**	45	80.7
8	DMT/NMPip/TfO$^-$	**5h**	30	78.6
9	DMT/NMPyr/TsO$^-$	**5i**	30	94.0
10	DMT/NMPyr/CsO$^-$	**5j**	45	87.2
11	DMT/NMPyr/MsO$^-$	**5k**	30	90.0
12	DMT/NMPyr/TfO$^-$	**5l**	30	64.0
13	**DMT/NMM/BF$_4^-$**		90	86

derived from *N*-hydroxybenzotriazole in the presence of a stoichiometric amount of DIPEA, the stability decreased with time, but not for COMU for which the degradation products were not observed under the same conditions. These observations have a practical implication both for solid-phase and solution strategies involving extremely poorly reactive carboxylic components, and for cyclisation steps proceeding slowly under high dilution conditions.

The effects of limited stability were, however, barely visible in the case of coupling of sterically hindered Z-Aib-OH, with H-Aib-OMe proceeding in dichloromethane (Table 5). Under such conditions, a 65–89% yield was obtained even with the less stable **5b**,**c** and **5g**. The most pure products were obtained with reagents derived from the weakly basic *N*-methylmorpholine **5a**–**d** and **5l**, although prolonged coupling time was required in the latter case.

Table 3. Stability of the sulfonates **5a**–**l**, DMT/NMM/BF_4^-,[15] TBTU, HBTU and COMU in DMF solution.

Coupling reagent	Stability [%][a] 1 h	6 h	12 h	1 d	2 d	4 d	7 d
5a	> 99	> 99	> 99	> 99	> 99	> 99	> 99
5b	> 99	> 99	> 99	> 99	> 99	> 99	> 99
5c	> 99	12	–	–	–	–	–
5d	> 99	> 99	> 99	> 99	> 99	> 99	> 99
5e	> 99	> 99	99	98	98	98	97
5f	> 99	> 99	> 99	98	98	97	97
5g	52	21	2.5	–	–	–	–
5h	> 99	> 99	> 99	99	98	97	96
5i	> 99	99	99	99	98	98	98
5j	> 99	> 99	> 99	> 99	> 99	> 99	> 99
5k	> 99	> 99	86	85	74	61	60
5l	> 99	> 99	> 99	99	99	99	99
TBTU	> 99	> 99	> 99	> 99	> 99	> 99	> 99
HBTU	> 99	> 99	> 99	> 99	> 99	> 99	> 99
COMU	> 99	> 99	> 99	> 99	> 99	> 99	> 99
DMT/NMM/BF_4^-	100	>99	>99	>99	>98	–	–

[a] Stability studies were performed by HPLC (Aqua C-18) column analysis of aliquots from stock solutions (0.25 M) of the various coupling reagents in DMF at indicated times, yields were calculated according to the integration of the peak area at 220 and 254 nm.

Table 4. Stability of sulfonates **5a**–**l**, DMT/NMM/BF_4^-,[15] TBTU, HBTU, and COMU in DMF in the presence of a stoichiometric amount of DIPEA.

Coupling reagent	Stability [%][a] 5 min	30 min	1 h	2 h	3 h
5a	> 99	> 99	> 99	> 99	> 99
5b	> 99	66	–	–	–
5c	> 99	> 99	15	11	–
5d	> 99	> 99	99	98	98
5e	> 99	> 99	> 99	> 99	> 99
5f	> 99	> 99	> 99	99	99
5g	97	88	37	35	35
5h	> 99	> 99	99	99	99
5i	> 99	99	99	99	99
5j	> 99	99	99	99	99
5k	> 99	98	98	93	93
5l	> 99	99	99	99	99
TBTU	> 99	> 95	> 90	> 90	> 80
HBTU	> 99	> 99	> 90	>80	> 70
COMU	> 99	> 99	> 99	> 99	> 99
DMT/NMM/BF_4^-	>99	>99	>99	>99	>99

[a] See footnote in Table 3.

Comparative studies on efficiency of other representative condensing reagents have shown that the application of COMU allows the final peptide to be obtained with comparable yield, but with moderate purity. On the other hand, in the case of TBTU, Z-Aib-Aib-OMe was obtained with moderate yield, but high purity.

The loss of chiral integrity is more severe during the activation of peptide fragments (even at the dipeptide stage) due to the open pathway leading to the formation of the epimerisation-prone oxazolone intermediate. Therefore, the ability of reagents **5a**–**l** to preserve the enantiomeric homogeneity in fragment condensation was carefully examined. The [2+1] coupling strategy in the synthesis of Z-Ala-Phe-Leu-OBz (**9**) was selected as a suitable peptide model to highlight the relative ability of the different TBCRs sulfonates **5a**–**l** to promote chirality retention. The activation of the epimerisation-prone C-terminal Phe provided a suitable scenario for evaluating the performance of the new coupling reagents described herein, in peptide fragment condensation strategy (Table 6).

Table 5. The synthesis of Z-Aib-Aib-OMe (**8a**–**l**) by using TBCRs **5a**–**l**, DMT/NMM/BF_4^-,[15] TBTU and COMU.

Entry	Dipeptide **8**	Coupling reagent	Activation time [h]	Yield [%]	Purity [%]
1	**8a**	**5a**	1	83	98.8
2	**8b**	**5b**	1	69	90.1
3	**8c**	**5c**	1	65	93.3
4	**8d**	**5d**	1	71	85.9
5	**8e**	**5e**	1	77	79.3
6	**8f**	**5f**	1	77	81.4
7	**8g**	**5g**	1	89	82.2
8	**8h**	**5h**	3	79	80.8
9	**8i**	**5i**	1	72	77.1
10	**8j**	**5j**	1	51	73.7
11	**8k**	**5k**	0.5	72	82.5
12	**8l**	**5l**	3	97	86.5
13	**8**	TBTU	1	75.5	96.8
14	**8**	COMU	1	95.8	81.2
15	**8**	DMT/NMM/BF_4^-	2.5	82	94.2

Table 6. Activation time, yield, purity and epimerisation in the synthesis of Z-Ala-Phe-Leu-OBz (**9a**–**l**) ([2+1] fragment condensation strategy) using TBCRs sulfonates **5a**–**l**.

Tripeptide **9**	Coupling reagent	Activation time [h]	Yield [%]	Purity [%]	Extent of epimerisation H_2N–X_1–X_2–X_3–COOH [%L]/[%D] X_1	X_2	X_3
9a	**5a**	1	88	99.5	100:0	100:0	99:1
9b	**5b**	2.5	63	97.3	100:0	100:0	100:0
9c	**5c**	1	74.4	97.4	100:0	100:0	100:0
9d	**5d**	1	72.5	95.5	100:0	100:0	100:0
9e	**5e**	1.5	78.6	99.0	98:2	98:2	100:0
9f	**5f**	1.5	53.4	98.7	100:0	100:0	95:5
9g	**5g**	1.5	81.5	89.4	99:1	100:0	99:1
9h	**5h**	3.5	80.2	95.2	100:0	97:3	99:1
9i	**5i**	1	81.6	99.1	99:1	100:0	100:0
9j	**5j**	1	75.3	99.3	100:0	100:0	100:0
9k	**5k**	0.5	71.2	97.8	100:0	100:0	100:0
9l	**5l**	3.5	77.3	90.1	100:0	95:5	98:2

It could be expected that in this type of coupling, the nature of the base and the rate of coupling would play a crucial role in preserving the chirality of the activated dipeptide starting material and the amino component. Importantly, the extent of racemisation of the Phe residue determined by GC on a ChirasilVal capillary column after hydrolytic degradation of the tripeptide **9** into amino acids, was maintained in all cases below 0.1% using all the TBCRs **5a**–**d**, derived from the weakly basic *N*-methylmorpholine.

On the other hand, prolonged coupling time was accompanied by partial epimerisation on all three stereogenic centres in the case of all *N*-methylpiperidine TBCRs **5e**–**h**. High levels of inversion of Phe and Leu configurations were also observed in the case of the TBCR **5l**, which was pre-

Table 7. Manual SPPS of the (65–74) ACP fragment **10** on 2-chlorotrityl resin using TBCRs **5a**–**l** and DMT/NMM/BF_4^-:[15] single coupling and a threefold excess of acylating reagents were used at all stages of the synthesis.

Coupling reagent	Condensation time [min] H_2N–V^1–Q^2–A^3-A^4–I^5–D^6-Y^7–I^8–N^9-G^{10}–2-chlorotrityl resin									Yield (purity) [%]
	V^1	Q^2	A^3	A^4	I^5	D^6	Y^7	I^8	N^9	
5a	15	15	15	15	15	15	15	15	30	97
5b	45	45	45	45	45	45	45	45	120	66
5c	30	30	30	30	30	30	30	30	30	82
5d	30	30	30	30	30	30	30	30	45	94
5e	30	30	30	30	30	30	30	30	45	92
5f	60	60	60	60	60	60	60	60	90	67
5g	15	15	15	15	15	15	15	15	30	82
5h	30	30	30	30	30	20	30	30	45	72
5i	15	15	15	15	15	15	15	15	30	83
5j	60	60	60	60	60	60	60	60	90	65
5k	15	15	15	15	15	15	15	30	30	89
5l	30	30	30	30	30	30	30	45	60	84
DMT/NMM/BF_4	15	15	15	15	15	15	15	15	15	84

pared from the relatively basic *N*-methylpyrrolidine and the strongly acidic trifluoromethanesulfonic acid **2d**. Interestingly, TBCRs **5h** and **5l**, prepared from **2d**, were substantially less reactive also in the tests involving coupling of sterically hindered substrates (Table 5). As expected, epimerisation in Leu, used as amino component, was observed only when a prolonged coupling time was required.

The next assay used to verify the usefulness of TBCRs **5a**–**l** in peptide bond formation was based on SPPS of the (65–74) ACP fragment (**10**) starting from Fmoc-Gly-2-chlorotrityl resin.

To emphasise the differences in terms of reactivity, we used a threefold excess of the acylating mixture in a single coupling procedure. The progress of all coupling reactions were monitored by Kaiser tests.[21] In particular, diverse reactivity of TBCRs **5a**–**l** in terms of time needed to complete each coupling step was observed. In fact, in the case of TBCR **5a**, the coupling reactions of each amino component proceeded considerably faster (15 min) than those reported in experiments performed with HATU (30 min) or TBTU (45 min) as commonly used coupling reagents under the same conditions.[15] Considering the relatively fast coupling process and satisfactory yield, methanesulfonates **5g**, **5i** and **5k** also appear to be extremely promising (see Table 7), although their very limited stability poses a major problem. The reagents prepared with 10-camphorsulfonic acid **5b**,**f**,**j** were less efficient due to the prolonged coupling time required and because of the relatively low yield of the final peptide.

Another useful test for rating the versatility of the new TBCRs involved the solid-phase synthesis of peptides containing difficult fragments because of the presence of sterically hindered α,α-disubstituted amino acid residues. Therefore, we prepared manually the [Aib]^4[Aib]^3-enkephalin analogue **11** on 2-chlorotrityl resin in a single coupling mode. The rate of coupling in each synthetic step was monitored with the Kaiser test. The purity of isolated products was estimated by standard chromatographic procedures. The obtained results showed the lowest yields (in the range of 79–83%) for the less stable TBCRs **5k** and **5g**, prepared from methanesulfonic acid and the relatively basic *N*-methylpyrrolidine and *N*-methylpiperidine, respectively. The highest yields (94–99%) were afforded by the coupling reagents **5a**–**c**, prepared from the less basic *N*-methylmorpholine (see Table 8). The latter results do not correlate with the stability tests (see Table 3 and Table 4) in the case of **5b**,**c**. However, these results are in compliance with the highest purity obtained in the case of the solution-phase synthesis of Z-AibAib-OMe. To explain this surprisingly poor correlation, it has to be taken into consideration that after a short preactivation time, the unstable TBCRs **5b**,**c** were transformed in a reaction with Fmoc-Aib-OH into the substantially more stable 4,6-dimethoxy-1,3,5-triazin-2-yl esters of appropriate *N*-protected amino acids. Thus, by using a freshly prepared DMF solution of *N*-triazinylammonium sulfonates, acceptable synthetic results could be obtained even in the case of the less stable TBCRs.

Table 8. Synthesis of the enkephalin analogue H_2N-Tyr-Aib-Aib-Phe-Leu-COOH (**11a**–**l**) with TBCR sulfonates **5a**–**l**.

Peptide **11**	Coupling reagent	Condensation time [h] H_2N-Tyr-Aib-Aib-Phe-Leu-2-chlorotrityl resin				Yield (purity) [%]
		Tyr	Aib	Aib	Phe	
11a	**5a**	0.5	1	1	0.5	99
11b	**5b**	0.5	1	2	1.5	97
11c	**5c**	0.5	0.5	0.5	1	94
11d	**5d**	0.5	1	2	0.75	85
11e	**5e**	0.5	0.75	1	1.5	87
11f	**5f**	0.5	1	2	0.5	92
11g	**5g**	0.5	1	1	2	83
11h	**5h**	0.5	0.75	1	0.5	98
11i	**5i**	0.5	1	0.75	2	91
11j	**5j**	0.5	1.5	2	1	94
11k	**5k**	0.5	1	2	0.75	79
11l	**5l**	0.5	0.75	0.5	0.75	86

In comparative studies using DMT/NMM/BF_4^-,[15] coupling proceeded less readily. The assembly of the first Fmoc-Aib-OH to the growing peptide chain on the resin was completed within 2 h, but incorporation of the second Fmoc-Aib-OH required extension of condensation time up to 4 h. The observed decrease of coupling rate in incorpora-

tion of lipophilic and sterically hindered Fmoc-Aib-OH into the peptide chain could be due to the nature of the counterion because in both cases the structure of the activated intermediate was identical.

Conclusions

There are several reports presenting the general opinion that the nature of the counteranion has no practical influence on the outcome of coupling reactions mediated by uronium/ammonium tetrafluoroborates or hexafluorophosphates.[22a] The preliminary results of our research on the efficiency of TBCR tetrafluoroborates[15] and hexafluorophosphates[22b] were also in agreement with this suggestion. However, the present study reporting the diversified stability and reactivity of the novel TBCR sulfonates **5a**–**l** surprisingly contradicts that. As a consequence, there is a need to re-evaluate this conclusion. The current comparative study using TBCRs **5a**–**l** derived from the three tertiary amines **3a**–**c** and four sulfonic acids **2a**–**d** with very diverse basic/acid, lipophilic/hydrophilic, and polarisation characteristics, yielding exactly the same structure of the reactive intermediate **7** after the activation of the carboxylic function, documented significant differences in stability, tendency to epimerisation, coupling rate, yield, and purity of the final products. The differences in synthetic versatility was noted both in the syntheses proceeding in solution as well as in manual SPPS. The TBCR (DMT/NMM/TsO$^-$, **5a**) prepared with the highly polarisable *p*-toluenesulfonate anion and the less basic *N*-methylmorpholine was found to be the most universal, and the coupling results surpassed those of its tetrafluoroborate analogue (DMT/NMM/BF_4^-). An additional, very important advantage of DMT/NMM/TosO$^-$ (**5a**) is its crystalline state, high stability (which allows long-term storage and shipping), as well as stability in solution under the conditions of SPPS. If the advantageous effects of polarisability of salt components are also observed in the case of other salt-type coupling reagents, a new pathway to improve the effectivness of several classical coupling reagents will be opened. This is particularly important for the fascinating area dedicated to syntheses of difficult peptides containing highly functionalised or noncoded amino acids.

Experimental Section

General Information: Thin-layer chromatography experiments (TLC) were carried out on silica gel (Merck; 60 Å F254), and spots were located with UV light (254 and 366 nm) and with 1% ethanolic 4-(4-nitrobenzyl)pyridine (NBP).

Analytical RP-HPLC was performed with a Waters 600S HPLC system (Waters 2489 UV/Vis detector, Waters 616 pump, Waters 717 plus autosampler, HPLC manager software from Chromax) using a Vydac C18 column (25 cm × 4.6 mm, 5 mm; Sigma). HPLC was performed with a gradient of 0.1% TFA in H_2O (A) and 0.08% TFA in MeCN (B), at a flow rate of 1 mL/min with UV detection at 220 nm, t_R in min; or on a Merck/Hitachi HPLC system (LaChrome; L-6200 pump, L-4000 UV detector, D-6000 interface, D-7000 HPLC manager) using a Supelco Discovery BIO Wide Pore C18 column (25 cm × 4.6 mm, 5 mm, Sigma). MS spectra were recorded with an IonSpec Ultima 4.7-T-FT ion cyclotron resonance spectrometer (ICR. HR-MALDI, in 2,5-dihydroxybenzoic acid matrix). LC/MS spectra were recorded with a Dionex UltiMate 3000. IR spectra were recorded as KBr pellets or film with a Bruker ALPHA spectrometer or a PerkinElmer Spectrum 100. ^1H and ^{13}C NMR spectra were recorded with a Bruker Avance DPX 250 (250 MHz) spectrometer or a Varian (300 MHz). Chemical shifts (ppm) are relative to TMS used as an internal standard. Multiplicities are marked as s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, m = multiplet. Melting points were determined with a Büchi apparatus, model 510.

Gas chromatography (GC) was conducted with a Shimadzu GC-14A, FID (H_2/air), split 1:50. Column: Chirasil-Val capillary (25 m × 0.32 mm), film thickness 0.2 μm, carrier gas: helium, pressure 0.45 atm. Temperature program: 4 min at 90 °C, next 4 °C/min to 190 °C, and 3 min at 90 °C.

Hydrolysis of Coupling Products to Amino Acid (GP 1): Peptide (5 mg) was treated with redistilled, constant-boiling HCl in a sealed tube at 100 °C for 20 h. The solution was concentrated to dryness, and the residue was dissolved with redistilled water and concentrated again. The remaining salt was dried overnight in a vacuum dessicator under P_2O_5 and then treated for 12 h at room temperature with anhydrous methanol saturated with dry HCl (2 mL). Methanol was evaporated, the residue was dried overnight in a vacuum dessicator under P_2O_5, then suspended in dichloromethane and treated with trifluoroacetic acid anhydride (50 μL) for 12 h at room temperature. The solution was analysed on a Chirasil-Val capillary column (25 m × 0.25 mm) with a split of 1:50; helium was used as carrier gas.

Manual Solid-Phase Peptide Synthesis (SPPS): Protected amino acids were purchased from Novabiochem or Bachem. All peptides were synthesised in an appropriate glass reactor or syringe by Fmoc methodology.

Loading of the 2-Chlorotrityl Chloride Resin (GP 2): The amino acid (3 equiv. rel. to the resin) and DIPEA (6 equiv.) were dissolved in CH_2Cl_2 (10 mL per 1 g of the resin), containing, if necessary, a small amount of DMF to facilitate dissolution of the amino acid. The 2-chlorotrityl chloride resin was preswollen in CH_2Cl_2 for 1 h, and then the solution containing the protected amino acid was added and the resin was shaken for 30–120 min. The resin was washed with CH_2Cl_2/MeOH/DIPEA (17:2:1, 3×), then DMF (2×) and CH_2Cl_2 (3×).

Standard Coupling Procedure (GP 3): Protected amino acid (3 equiv.), **5** (3 equiv.) and DIPEA (6 equiv.) were mixed and added to the resin. The resin was shaken for 1–2 h. The progress of the reaction was monitored by Kaiser[21] or TNBS tests.[23]

Deprotection (GP 4): The Fmoc protecting group was removed by treatment with a solution of 20% piperidine in DMF (2× 5 min).

Cleavage from the Resin (GP 5): The peptides were cleaved from the resin by using TFA/Et_3SiH/H_2O, 9.5:2.5:2.5 (ca. 2 mL/0.1 g of resin). Cleavage was performed during 4 h, then the resin was filtered off, and the filtrate was evaporated. To the oily residue, Et_2O was added to precipitate the peptide. The resulting solid was filtered off, washed with Et_2O, dried and lyophilised.

4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium Toluene-4-sulfonate (DMT/NMM/TosO-5a); Typical Procedure: To a vigorously stirred solution of 4-methylmorpholinium toluene-4-sulfonate (5.46 g, 20 mmol) and sodium hydrogen carbonate

(5.04 g, 60 mmol) in acetonitrile (60 mL), cooled to 5 °C, 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT) (3.50 g, 20 mmol) was added. The mixture was stirred at 5 °C for 20 h and the progress of the reaction was monitored by TLC (staining with 0.5% solution of NBP in EtOH). Upon complete consumption of CDMT, the precipitate was filtered off, and the filtrate was evaporated to dryness at a temperature not exceeding 20 °C. The solid residue was washed with THF and recrystallised from acetonitrile/diethyl ether to afford **5a** (5.93 g, 72%); m.p. 57–61 °C. IR (film/NaCl): $\tilde{\nu}$ = 3418, 3041, 2957, 2111, 1630, 1540, 1483, 1457, 1408, 1386, 1338, 1319, 1308, 1285, 1271, 1227, 1198, 1132, 1122, 1060, 1037, 1014 cm^{-1}. ^1H NMR (300 MHz, CD_3CN): δ = 2.35 (s, 3 H, CH_3-C_6H_4-), 3.42 (s, 3 H, CH_3-N), 3.73–4.03 (m, 6 H, -*N*-CH_2-CH_2-O-), 4.12 (s, 6 H, 2 × CH_3-O), 4.20–4.48 (m, 2 H, -*N*-CH_2-CH_2-O-), 7.22 (d, *J* = 7.5 Hz, 2 H, -C_6H_4-), 7.62 (d, *J* = 7.5 Hz, 2 H, -C_6H_4-) ppm. ^{13}C NMR (75 MHz, CD_3CN): δ = 22.7, 55.6, 58.2, 59.2, 62.4, 128.0, 131.1, 142.5, 145.3, 172.6, 176.3 ppm. HRMS: *m*/*z* calcd. for $C_{17}H_{25}N_4O_6S^+$ 413.48; found: 413.1490 [M^+], 414.1525 [M + H]$^+$. $C_{17}H_{24}N_4O_6S$ (413.47): calcd. C 49.50, H 5.87, N 13.58, O 23.27, S 7.77; found C 49.51, H 5.86, N 13.58, S 7.77.

4,6-Dimethoxy-1,3,5-triazin-2-yl 4-Methoxybenzoate (7); Typical Procedure: 4-Methoxybenzoic acid (1 mmol) and DIPEA (88 μL, 0.5 mmol) were added at 0 °C to a vigorously stirred solution of **5** (1 mmol) in CH_2Cl_2 (3 mL). Stirring was continued until the disappearance of condensing reagent **5** (TLC analysis, staining with 0.5% solution of NBP), after which time the mixture was diluted with CH_2Cl_2 (10 mL), and the solution was washed successively with water, 0.5 M aqueous $KHSO_4$, water, 0.5 M aqueous $NaHCO_3$, and water again. The organic layer was dried with $MgSO_4$, filtered, and concentrated to dryness. The residue was dried under vacuum with P_2O_5 and KOH to constant weight.

4,6-Dimethoxy-1,3,5-triazin-2-yl 4-Methoxybenzoate (7a): Obtained from 4-methoxy-benzoic acid (0.152 g, 1 mmol), DIPEA (88 μL, 0.5 mmol), and DMT/NMM/TsO^- (**5a**) (0.413 g. 1 mmol), in 30 min, yield 0.287 g (98.5%). IR (film/NaCl): $\tilde{\nu}$ = 3461, 3078, 3023, 2953, 2844, 2604, 2337, 2172, 2072, 1945, 1759, 1744, 1590, 1570, 1544, 1512, 1470, 1444, 1427, 1406, 1355, 1318, 1242, 1233, 1202, 1189, 1172, 1164, 1108, 1085, 1042, 1021 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ = 3.76 (s, 3 H, CH_3O-C_6H_4-), 3.97 (s, 6 H, CH_3O-CN), 6.84 (d, *J* = 7.5 Hz, 2 H, C_6H_4-), 7.98 (d, *J* = 7.5 Hz, 2 H, C_6H_4-) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 55.6, 55.9, 113.8, 119.9, 132.6, 163.8, 170.1, 173.6 ppm. LC/MS: *m*/*z* calcd. for $C_{13}H_{13}N_3O_5^+$ 291.27; found 292.1 [M + H]$^+$.

Z-Aib-Aib-OMe (8); Typical Procedure: Z-Aib-OH (0.237 g, 1 mmol) and DIPEA (0.088 mL, 0.5 mmol) were added to a vigorously stirred solution of **5** (1 mmol) in CH_2Cl_2 (5 mL), cooled to 0 °C. Stirring was continued until the disappearance of condensing reagent **5** was observed (TLC analysis, staining with 0.5% solution of NBP), after which time HCl·Aib-OMe (0.154 g, 1 mmol) and DIPEA (0.176 mL, 1 mmol) were added, and the mixture was stirred for an additional 2 h at 0 °C and overnight at room temperature. The mixture was diluted with CH_2Cl_2 (10 mL), then the solution was washed successively with water, 0.5 M aqueous $NaHSO_4$, water, 0.5 M aqueous $NaHCO_3$, and water again. The organic layer was dried with $MgSO_4$, filtered, and concentrated to dryness. The residue was dried under vacuum with P_2O_5 and KOH to constant weight, to afford the neutral peptide.

Z-Aib-Aib-OMe (8a): Obtained from Z-Aib-OH (0.237 g, 1 mmol), HCl·Aib-OMe (0.154 g, 1 mmol), DMT/NMM/TsO^- (**5a**) (0.413 g, 1 mmol), and DIPEA (0.264 mL, 1.5 mmol). Activation time: 1 h, yield 0.279 g (83%). IR (film/NaCl): $\tilde{\nu}$ = 3380, 3362, 3315, 3276, 3033, 2985, 2939, 1726, 1713, 1655, 1517, 1453, 1409, 1385, 1363, 1299, 1251, 1226, 1193, 1170, 1152, 1087, 1066, 1021 cm^{-1}. ^1H NMR (250 MHz, $CDCl_3$): δ = 1.52 (s, 12 H, CH_3), 3.72 (s, 3 H, CH_3-O-), 5.10 (s, 2 H, Ph-CH_2-O), 5.22–5.30 (m, 1 H, -CO-NH), 6.89–6.91 (m, 1 H, CO-NH-), 7.29–7.40 (m, 5 H, ArH) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 24.4, 25.2, 52.5, 56.9, 65.4, 67.0, 128.0, 128.1, 128.5, 156.2, 171.6, 174.0, 176.5 ppm. LC/MS: *m*/*z* calcd. for $C_{17}H_{24}N_2O_5^+$ 336.39; found 337.3 [M + H]$^+$. Anal. RP-HPLC (3–97%B in 30 min): t_R = 9.55 min (purity 98.8%).

Z-Ala-Phe-Leu-OBz (9); Typical Procedure: Z-Ala-Phe-OH (0.370 g, 1 mmol) and DIPEA (0.088 mL, 0.5 mmol) were added to a vigorously stirred solution of **5** (1 mmol) in CH_2Cl_2 (5 mL), cooled to 0 °C. Stirring was continued until the disappearance of a condensing reagent **5** was observed (TLC, staining with 0.5% solution of NBP), after which time TsOH·Leu-OBz (0.393 g, 1 mmol) and DIPEA (0.176 mL, 1 mmol) were added, and stirring was continued for an additional 2 h at 0 °C and overnight at room temperature. The mixture was diluted with CH_2Cl_2 (10 mL) and the solution was washed successively with water, 0.5 M aqueous $NaHSO_4$, water, 0.5 M aqueous $NaHCO_3$, and water again. The organic layer was dried with $MgSO_4$, filtered, and concentrated to dryness. The residue was dried under vacuum with P_2O_5 and KOH to constant weight to afford Z-Ala-Phe-Leu-OBz.

Z-Ala-Phe-Leu-OBz (9a): Obtained from Z-Ala-Phe-OH (0. 0.370 g, 1 mmol), TsOH·Leu-OBz (0.393 g, 1 mmol), DMT/NMM/TsO^- (**5a**) (0.413 g, 1 mmol), and DIPEA (0.264 mL, 1.5 mmol). Activation time: 1 h, yield 0.505 g (88%). IR (film/NaCl): $\tilde{\nu}$ = 3276, 3068, 3033, 2956, 2871, 2035, 1950, 1738, 1687, 1644, 1533, 1484, 1453, 1386, 1368, 1326, 1255, 1234, 1192, 1146, 1113, 1075, 1028 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$): δ = 0.91 [d, *J* = 6.5 Hz, $(CH_3)_2CH$, 6 H], 1.47–1.49 [m, 1 H, $(CH_3)_2CH$-], 1.48 (d, *J* = 7.2 Hz, 3 H, CH_3-CH-), 1.82–1.89 (m, 2 H, -CH-CH_2-), 3.19, 3.44 (dd, J_1 = 8.3, J_2 = 4.1 Hz, 2 H, -CH-CH_2-), 4.41 (t, *J* = 5.2 Hz, 1 H, -CH_2-CH-), 4.68 (q, *J* = 7.2 Hz, 1 H, CH_3-CH-), 4.80–4.88, (m, 1 H, -CH-CH_2-), 5.09 (s, 2 H, -CH_2-Ph), 5.34 (s, 2 H, -CH_2-Ph), 7.25–7.40 (m, 15 H, Ph) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 18.5, 21.8, 22.6, 24.6, 38.1, 40.8, 41.9, 50.4, 53.9, 54.1, 66.8, 126.7, 127.8, 128.0, 128.1, 128.3, 128.4, 135.3, 136.1, 136.3, 155.8, 170.5, 172.1, 172.3 ppm. Anal. RP-HPLC (3–97%B in 30 min): t_R = 23.82 min (purity 99.5%). LC/MS: *m*/*z* calcd. for $C_{33}H_{39}N_3O_6^+$ 573.70; found 574.4 [M + H]$^+$. GC (hydrolyzate was derivatised according to GP 1; chromatography conditions: temp. 90 °, 4 min, 90–200 °, 4 °/min, 200 °, 3 min) t_R = 3.07 (D-Ala), 3.13 (L-Ala) min L/D = 100:0; t_R = 17.27 (L-Phe), 17.38 (D-Phe) min L/D = 100:0; t_R = 6.56 (D-Leu), t_R 6.91 (L-Leu) min L/D = 99:1.

Synthesis of (65–74) ACP: H_2N-Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly-OH (10): 2-Chlorotrityl chloride resin (600 mg, 1.1 mmol/g, 0.66 mmol) was esterified with Fmoc-Gly-OH (0.589 g, 1.98 mmol) in the presence of DIPEA (0.713 mL, 3.96 mmol) according to GP 2, followed by Fmoc deprotection (GP 4). Modified resin was divided into 12 portions (50 mg, 0.055 mmol) and used for further reaction steps in separate reactors. Subsequently, the peptide chains were elongated (GP 3), respectively, with Fmoc-Asn(Trt)-OH (98 mg, 0.165 mmol), Fmoc-Ile-OH (58 mg, 0.165 mmol), Fmoc-Tyr(*t*Bu)-OH (76 mg, 0.165 mmol), Fmoc-Asp(*t*Bu)-OH (68 mg, 0.165 mmol), Fmoc-Ile-OH (58 mg, 0.165 mmol), Fmoc-Ala-OH (51 mg, 0.165 mmol), Fmoc-Ala-OH (51 mg, 0.165 mmol), Fmoc-Gln(Trt)-OH (101 mg, 0.165 mmol) and Fmoc-Val-OH (56 mg, 0.165 mmol) in the presence of appropriate reagent **5a**–**l** (0.165 mmol) and DIPEA (60 μL, 0.33 mmol). After the last deprotection (GP 4), the peptides were cleaved from the resin (GP 5).

Compound 10a: Coupling reagent: DMT/NMM/TsO^- (**5a**) (68 mg, 0.165 mmol) at each step of an elongation peptide chain. Product:

10a: Anal. RP-HPLC (3–97%B in 30 min): t_R = 22.91 min (purity 97.9%). LC/MS: *m*/*z* calcd. for $C_{47}H_{74}N_{12}O_{16}^+$ 1063.18; found 1064.5 $[M + H]^+$.

H_2N-Tyr-Aib-Aib-Phe-Leu-OH (11): 2-Chlorotrityl chloride resin (600 mg, 1.1 mmol/g, 0.66 mmol) was esterified with Fmoc-Leu-OH (0.700 g, 1.98 mmol) in the presence of DIPEA (0.713 mL, 3.96 mmol) according to GP 2, followed by Fmoc deprotection (GP 4). Modified resin was divided into 12 portions (50 mg, 0.055 mmol) and used for the further reaction steps in separate reactors. Subsequently, the peptide chains were elongated (GP 3), respectively, with Fmoc-Phe-OH (64 mg, 0.165 mmol), Fmoc-Aib-OH (54 mg, 0.165 mmol), Fmoc-Aib-OH (54 mg, 0.165 mmol) and Fmoc-Tyr(*t*Bu)-OH (76 mg, 0.165 mmol) in the presence of **5a**–**l** (0.165 mmol) and DIPEA (60 μL, 0.33 mmol). After the last deprotection (GP 4), the peptides were cleaved from the resin (GP 5).

Compound 11a: Coupling reagent: DMT/NMM/TsO$^-$ (**5a**) (68 mg, 0.165 mmol) at each step of an elongation peptide chain. Product: **11a**: Anal. RP-HPLC (3–97%B in 30 min): t_R = 16.81 min (purity 99.5%). LC/MS: *m*/*z* calcd. for $C_{32}H_{45}N_5O_7^+$ 611.74; found 612.4 $[M + H]^+$.

Supporting Information (see footnote on the first page of this article): Synthetic procedures for **5b**–**l**, chromatographic characterisation of products as well as ^{1}H NMR, ^{13}C NMR, MS and IR spectra.

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